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Faculdade de Medicina

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Ivonne Wollenberg

Ph.D. thesis in Biomedical Science

(Speciality Immunology)

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Abbreviation List

Ab – antibody

AHR - airway hyperreactivity

AID - activation-induced cytodine deaminase

APC – antigen presenting cell

BCR – B cell receptor

C – constant (region)

CCR7 – C chemokine receptor 7

D – diversity (gene segment)

CD – cluster of differentiation

CDR – complementary determining regions

CSR – class switch recombination

CTLA-4 - cytotoxic T cell associated antigen-4

CXCR5 – CX chemokine receptor 5

DC – dendritic cell

FDC – follicular dendritic cell

FR – framework regions

FRC – fibroblastic reticular cells

GC – germinal center

GCR – germinal center reaction

H – heavy (chain)

ICOS – inducible T cell costimulator

iTreg – induced regulatory T (cell)

Ig – immunglobulin

IFN – interferon

IL – interleukin

i.p. – intraperitoneal

i.v. – intravenous

J – joining (gene segment)

L – light (chain)

Lck – lymphocyte-specific protein tyrosin kinase

LPS - lipopolysaccharide

ko –knockout

LN – lymph node

mAb – monoclonal antibody

mLN – mesenteric lymph node

MHC – major histocompatibility complex

mTEC – medullary thymic epithelial cell

NK – natural killer (cell)

NKT – natural killer T (cell)

nTreg – natural regulatory T (cell)

OVA – ovalbumin

PAMPs – pathogen associated molecular patterns

PD-1 – programmed death-1

PRR – pattern recognition receptor

SHM – somatic hypermutation

SLO – secondary lymphoid organs

SPF – specific pathogen free

Src - sarcoma

TCR – T cell receptor

TD – T cell dependent

TdT – terminal desoxyribonucloetidyl transferase

T_{FH} – follicular helper T (cell)

$T_F$reg – follicular regulatory T (cell)

Th – T helper (cell)

TI – T cell independent

TLR – Toll like receptor

TNFRSF – TNF receptor superfamily

Treg – regulatory T (cell)

V- variable (region)

VL – variable light (chain)

VH – variable heavy (chain)

Sumário

Esta tese descreve o estudo da regulação de respostas imunitárias que conduzem à produção de anticorpos. Este tipo de respostas imunitárias depende de interações T-B. A primeira parte da tese descreve o papel do bloqueio do ligando do OX40 (OX40L) na prevenção do desenvolvimento da asma alérgica num modelo animal. A asma alérgica é uma patologia dependente de células Th2 associada à produção de IgE e IgG1. A segunda parte desta tese descreve a regulação da reacção dos centros germinativos, um evento chave na produção de anticorpos e células B de memória. Este estudo levou à identificação de uma população funcionalmente relevante de células T foliculares com fénotipo regulador, isto é, células que expressam o factor de transcrição Foxp3 para além dos marcadores característicos de células T foliculares (PD-1, CXCR5 e Bcl-6).

A produção de anticorpos nos tecidos linfóides secundários, tal como os gânglios linfáticos e baço, em resposta a antigénios requer a rápida expansão de células CD4 específicas para esse antigénio e o seu recrutamento para os locais onde vão colaborar com as células B. Na zona T, as células T providenciam ajuda às células B, permitindo a rápida formação de plasmócitos em locais extrafoliculares. Nos folículos linfóides, as células T CD4 são necessárias para o desenvolvimento dos centros germinativos, importantes para a formação dos linfócitos B de memória e dos precursores de plasmócitos. Além disso, após re-exposição ao mesmo antigénio, as células T de memória fornecem ajuda às células B, tanto de memória como naïve, de modo a obter uma resposta secundária mais rápida. As interacções celulares e moleculares que direccionam as células T para auxiliarem as células B durante uma resposta humoral, bem como a sua regulação, ainda não são bem compreendidas. O sinal co-estimulatório fornecido pelas células dendríticas através de CD28 às células T é essencial ao desenvolvimento dos centros germinativos. As células CD4 activadas com CD28 passam a expressar OX40, uma molécula que não é expressa nas células T naïve, o que permite a obtenção de sinais secundários através de OX40L. Esta molécula é expressa nas células dendríticas activadas por CD40. Foi descrito que OX40 promove o desenvolvimento de células Th2 e a expressão do receptor de quimiocina CXCR5, que direcciona a migração de células CD4 para os folículos linfóides onde o seu ligando é expresso.

Neste trabalho examinámos o papel do OX40L no desenvolvimento da inflamação alérgica das vias aéreas, mediada por células Th2, usando um anticorpo monoclonal que bloqueia OX40L. A sensibilização e re-exposição intra-nasal com ovalbumina em ratinhos BALB/c induz características típicas da asma alérgica, nomeadamente a hiperreactividade das vias aéreas, infiltrados eosinofílicos, hiperplasia das células caliciformes e produção de citocinas Th2 nos pulmões. Observámos que a administração do anticorpo monoclonal bloqueante anti-OX40L preveniu a indução da inflamação das vias aéreas. No entanto, este tratamento não levou à indução de tolerância específica para o antigénio administrado. Estes resultados mostram que OX40L tem um papel importante na fase de indução da doença.

Recentemente, a expressão de CXCR5, PD-1 e do factor de transcrição Bcl-6 permitiu a identificação de uma subpopulação de células T especializadas em providenciar ajuda às células B nos folículos linfóides. Estas células foram denominadas de células T auxiliares do folículo (T_{FH}). As células T_{FH} participam na resposta humoral providenciando sinais importantes para a ocorrência de hipermutação somática e maturação da afinidade das células B dos centros germinativos.

Encontrámos uma sub-população de células T foliculares, com características fenótipicas de células T_{FH} que co-expressam Foxp3 e que são recrutadas durante a reacção do centro germinativo. Mostrámos que estas células T foliculares Foxp3$^+$ derivam da população de células T reguladoras naturais. Com o propósito de estabelecer a importância fisiológica das células T foliculares Foxp3$^+$ *in vivo*, usámos células Foxp3$^+$ deficientes em CXCR5, que deste modo não conseguem aceder à região folicular. A transferência destas células Foxp3$^+$ deficientes em CXCR5 mostrou que as células T foliculares Foxp3$^+$ são importantes na regulação da reacção do centro germinativo depois da imunização com um antigénio T-dependente. Os nossos resultados *in vivo*s mostraram que as células T foliculares Foxp3$^+$ podem limitar a magnitude da reacção do centro germinativo, bem como a quantidade secretada de IgM, IgG1, IgG2b e IgA específicas para o antigénio. Como tal, as células T foliculares Foxp3$^+$ parecem combinar características das células T auxiliares do folículo, com as características das células T reguladoras para controlar as respostas imunes humorais.

No seu conjunto, os dados desta tese descrevem a identificação de mecanismos chave na regulação da reacção do centro germinativo que, em última análise, previnem as patologias mediadas por anticorpos.

Abstract

This thesis reports research on the regulation of immune responses leading to a humoral immune reaction. This type of immune phenomena is based on B-T cell interactions. The first part of the thesis is devoted to study the effect of OX40-ligand blockade in preventing allergic airways disease in mice. Allergic airways disease is a Th2-dependent pathology associated with production of IgE and IgG1 specific to the allergen. In the second part of the thesis the regulation of germinal centre reaction, a key event for the production of antibodies and B cell memory, is investigated leading to the identification of a follicular population of $Foxp3^+$ regulatory T cells.

To mount a successful antibody response to antigens, rare antigen-specific CD4 T cells have to be recruited to the secondary lymphoid tissues, enabling cognate B cell/T cell interactions. In the T cell zone, T cells provide help for B cells, allowing first a fast antibody response by the generation of plasma cells in extrafollicular foci. In the B cell zone, CD4 T cells are required for the development of germinal centers, which subsequently give rise to memory B cells and the precursors of long-lived plasma cells. Moreover, after re-exposure to antigen, memory CD4 T cells provide help to both memory and naive B cells for more efficient secondary immune responses. The cellular and molecular interactions that direct T cells to encounter with B cells for cognate interaction to provide survival and differentiation signals in secondary immune responses, are still incompletely understood. The CD28 costimulatory signal that T cells receive from dendritic cells, during priming in the T cell zone, are essential for GC development. Activation of CD4 T cells through CD28 upregulates OX40, which is not expressed on naive T cells, allowing CD40-activated dendritic cells to provide secondary signals through OX40 ligand. It has been reported that OX40 signals can promote Th2 development and induce expression of the chemokine receptor CXCR5 by CD4 T cells, which directs their migration to B cell follicles following a CXCL12 gradient, which is the ligand of CXCR5.

We examined the role of OX40L in the development of Th2-mediated airway inflammation by utilizing a blocking anti-OX40L monoclonal antibody (mAb). Sensitization and airway challenge with ovalbumin in BALB/c mice induced typical features of allergic asthma, namely airway hyperreactivity, eosinophilic infiltrates in the airways, hyperplasia of goblet cells with increased mucus production, and high levels of Th2 cytokines in the lung. Administration of blocking anti-OX40L mAb at the time of sensitization prevented the induction of airways inflammation. However, treatment with anti-OX40L mAb did not lead to long-term tolerance against the administered allergen. These results indicate a critical role for OX40L in the induction phase, which leads to the development of pathogenic Th2 cells, but not in the induction of tolerance.

Recently, expression of CXCR5, PD-1, and the transcription factor Bcl-6 has allowed the identification of a defined T cell subpopulation specialized in providing B cell help in lymphoid follicles. These cells have been named follicular helper T cells (T_{FH}). T_{FH} cells

participate in humoral responses providing signals required for somatic hypermutation and affinity maturation of germinal center B cells.

We found that a proportion of follicular T cells, with phenotypic characteristics of T_{FH} cells but expressing Foxp3 are recruited into the germinal center during the course of a germinal centre reaction. We found that, these follicular Foxp3$^+$ T cells derive from natural regulatory T cells. In order to establish the *in vivo* physiological importance of Foxp3$^+$ follicular T cells we used CXCR5-deficient Foxp3$^+$ cells, which do not have access to the follicular region. Adoptive cell transfers of CXCR5-deficient Foxp3$^+$ cells showed that Foxp3$^+$ follicular T cells are important regulators of the germinal center reaction following immunization with a thymus-dependent antigen. Our *in vivo* data show that Foxp3$^+$ follicular T cells can limit the magnitude of the germinal center reaction and also the amount of secreted antigen-specific IgM, IgG1, IgG2b and IgA. Therefore, Foxp3$^+$ follicular T cells appear to combine characteristics of follicular helper T cells and regulatory T cells for the control of humoral immune responses.

Taken together, the data in this thesis report the identification of key mechanisms regulating the germinal center reaction, and ultimately preventing antibody-mediated pathology.

1. General Introduction

1.1 The immune system

Early vertebrates evolved throughout millennia a complex immune system that protects individuals from a broad range of dangerous pathogens. It can be traditionally divided into innate and adaptive immune system, each with different function and role.

The innate immune response involves several different cellular players, as well as specific molecules, allowing a fast, but unspecific response, being the first line of defense against foreign pathogens. The adaptive immune response takes around 2-3 days to start, but has the advantage, that the response is specific for each pathogen and by that very effective. As a consequence, the adaptive immune response represents the second line of defense during an infection. Another main feature of the adaptive immune response is to generate memory cells after the encounter with a pathogen. These memory cells persist in the host under steady state conditions for months to years and allow the body to mount a fast specific response (secondary immune response) when exposed a second time to the same pathogen. This secondary immune response represents a powerful tool in the host defense against pathogens and is the basis for prophylactic vaccination.

1.1.1 The innate Immune Response

The effector mechanisms of innate immunity, which include antimicrobial peptides, phagocytes, and the alternative complement pathway, are activated immediately after infection to fast and efficiently control the infecting pathogen.

During evolution, the innate immune system appeared before the adaptive immune system, and some form of innate immunity probably exists in all multicellular organisms. This system consists of a humoral and a cellular part. The cellular part of the innate immune system is performed by cells of hematopoetic and nonhematopoetic origin. Hematopoetic cells involved in innate immunity include macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, natural killer (NK) cells and natural killer T (NKT) cells. Albeit DCs and NK T cells are classically seen as part of the innate immune system, they also show features of the adaptive immune system, hence they represent a bridge between innate and adaptive immune response. In addition to hematopoetic cells, innate immune response also includes nonhematopoetic cells like for example the epithelial cells of the skin. In contrast to the adaptive immunity, innate immune recognition is mediated by receptors with a genetically predetermined specificity (expressed mainly by macrophages and DCs). The advantage of these germ-line-encoded receptors is that they evolved to have defined specificities for common molecular structures represented by infectious microorganisms. The disadvantage is, that microorganisms can mutate at much higher rates than any of their hosts. Therefore the strategy of the innate immune response is to focus on a few, highly conserved structures present in large groups of microorganisms, rather than recognizing

every possible antigen (Janeway, 1989). These structures are referred to as pathogen-associated molecular patterns (PAMPs), and the receptors of the innate immune system that evolved to recognize them are called pattern-recognition receptors (PRRs). Examples of PAMPs are bacterial lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA, and glucans. Eventhough these molecules are chemically quite distinct, all PAMPs share certain features (Janeway, 1992; Medzhitov and Janeway, 1997). PAMPs are produced only by microbial pathogens, and not by their hosts. For example, LPS is synthesized only by gram-negative bacteria. Furthermore, the molecular structures recognized by the innate immune system are usually essential for the survival or pathogenicity of the microorganism, as a consequence those structures are more conserved between different species and cannot be easily altered to escape the immune defense. For example, all gram-positive bacteria have lipoteichoic acids, and therefore, the lipoteichoic acid PRR of the host can detect the presence of virtually any gram-positive bacterial infection. Signaling receptors recognize PAMPs and activate signal-transduction pathways that induce the expression of a variety of immune-response genes, including inflammatory cytokines. The receptors of the toll-like family (TLR) have a major role in this induction.

To support and increase these cellular defenses, innate immunity also has a humoral component. Those proteins, such as complement proteins, LPS binding protein, C-reactive protein and others circulate through the body and are involved in both sensing microbial components and effector mechanisms to facilitate clearance of infection.

1.1.2 The adaptive Immune Response

The adaptive component of the immune system is organized around two classes of specialized cells, T cells and B cells. The T-cell receptor (TCR) and the B-cell receptor (BCR) are, not like in the innate immune system germline-encoded, but rather generated somatically. Given the fact that these receptors are not genetically encoded, they are not predestined to recognize any particular antigen and therefore an extremely diverse repertoire of receptors has to be generated. Given that each lymphocyte displays a single kind of structurally unique receptor, that the number of lymphocytes is very large ($>10^8$ in mice) and that the average lymphocyte clonal size is small (<100 cells), it follows that the repertoire of antigen receptors in the entire population of lymphocytes is very large and extremely diverse. The dimension and diversity of this repertoire increases the possibility that an individual lymphocyte will encounter an antigen that binds to its receptor, leading to activation and proliferation of this cell.

As each T and B cell owns a unique kind of receptor, clonal expansion of lymphocytes in response to infection is absolutely mandatory for the generation of an efficient immune response. After having contact to a foreign pathogen the lymphocyte gets activated and goes through multiple rounds of proliferation building a clonal army against this specific pathogen. However, since the binding sites of the receptor arise from a random process, these binding sites also possess the risk of generating receptors that recognize self antigens.

1.2 B cells

In mammals, B cells arise from hematopoetic cells in the bone marrow or fetal liver and achieve maturity in peripheral lymphoid organs. In the bone marrow of adults B cells pass through several distinct developmental stages, during which they acquire their antigen-specificity. At an immature stage, B cells exit the bone marrow and complete their development to the mature or naive stage in the periphery. Those naive B cells express IgM together with IgD on their surface. The development in the bone marrow occurs in the absence of any exogenous antigen. Thus this stage is called antigen-independent B-cell development (Fig. 1).

After maturing in the secondary lymphoid organs (SLOs) naive, mature follicular B cells recirculate through the body and migrate repeatedly through the B cell area of the spleen and the lymph nodes, waiting to get activated as a consequence of antigen recognition.

In the lymph nodes B cells are concentrated in the cortex in primary follicles, in contact with follicular dendritic cells (FDC). B cells, unlike T cells, recognize antigens in their native form. Low-molecular-weight antigens might diffuse directly into B-cell areas in secondary lymphoid tissues. Larger molecules require active cellular mechanisms that are still being examined (Batista and Harwood, 2009; Cyster, 2010). Antigens complexed with IgM, IgG, and complement might be carried on the surfaces of specialized macrophages, FDCs and even B cells themselves, collectively expressing receptors for IgG Fc and complement fragments on their surface (Elgueta et al., 2010). Antigen presented on the surface of these cells can stimulate B cells through B cell receptor (BCR) crosslinking, expression of other interacting surface molecules, and secreted cytokines.

The surface immunglobulin that serves as the BCR has two functions in B-cell activation. First, like the antigen receptor on T cells, it transmits signals directly to the cell's interior when it binds antigen. Second, the BCR delivers the antigen to intracellular sites where it is processed and subsequently returned to the B-cell surface as peptide bound to major histocompatibility complex (MHC) class II molecules. B cells require two principal types of signals to become activated. Signal 1 is delivered by cross-linking of the immunoglobulin receptor. This cross-linking leads to activation of intracellular signaling pathways allowing the cell to interact with T cells that recognize the same antigen and thus deliver the signal 2 through CD40.

The cognate interaction between T cells and B cells is analogous to the interaction between T cells and DCs, which are usually referred to as professional antigen presenting cells (APCs). B cells express many of the same costimulatory molecules found on DCs, such as CD40, B7-1 (CD80), and B7-2 (CD86). T cells and B cells form an analogous immunologic synapse, which is followed by a signaling pathway. This initial interaction takes place at the boundary between primary follicles and T-cell areas in SLOs. The activated B cells enter one of two pathways: either they immediately start secreting low-affinity antibodies as short lived plasma cells, or they enter a follicle to establish a germinal center (GC) (Batista and Harwood, 2009). Some microbial antigens can activate B cells directly in the absence of T cell

help. The ability of B cells to respond directly to these antigens provides a rapid response to many important bacterial pathogens. However, somatic hypermutation and switching to certain immunoglobulin isotypes depends on the interaction of antigen-activated B cells with helper T cells.

B cells are active as APCs and express peptides along with MHC class II on their surface. These peptides, as already mentioned above, originate from processed antigen internalized after binding to the BCR (Huston, 1997). When the B cell contacts a $CD4^+$ T cell specific for such a MHC class II/peptide complex and having been previously activated by a professional APC, the T cell is able to provide cognate help and activate the B cell for further differentiation into memory or plasma cell.

1.2.1 B cell subpopulations

Newly formed immature B cells are defined by their short half-lives and their tendency to undergo apoptosis rather than proliferating following BCR engagement and are usually classified as transitional (T)1 and T2 B cells (LeBien and Tedder, 2008) (see Fig. 1). Naive mature B cells can be commonly divided into three subsets, B-1 B cells (that are typically subdivided into B-1a and B-1b), follicular B cells and marginal zone B cells. The cells of the different subsets vary in terms not only of their location, but also of their ability to migrate and likelihood to be activated in a T-dependent (TD) or T-independent (TI) fashion.

Marginal zone B cells are positioned at the marginal zone by the activity of S1P1 and S1P3, receptors for sphingosine1-phosphate (Cinamon et al., 2004; Cinamon et al., 2008; Vora et al., 2005) and have a particular role in responding to TI antigens type 2 (Steiniger et al., 2006). They are involved in TD B cell responses, can mediate the transport of antigen in form of immune complexes into splenic follicles, but they may also participate in immune responses to lipid antigens. Marginal zone B cells can be induced to differentiate into short-lived plasma cells in the absence of ligation through their BCR, hence they are considered to be innate-like B cells. It is to date not fully understood whether a distinct population of marginal-zone B cells also exists in humans, as the histological structure of the spleen is distinct from the murine one.

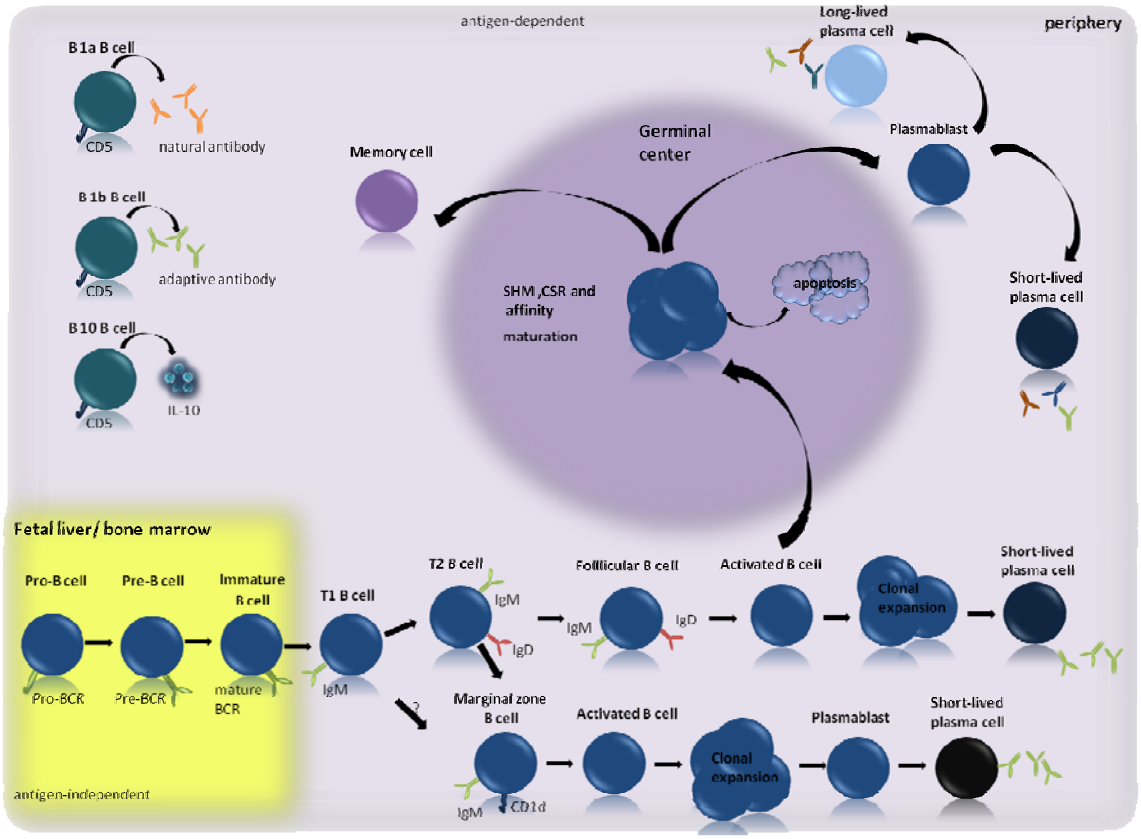

Figure1. B cell development and B cell subpopulations. B cell development starts in the fetal liver or bone marrow. The terminal differentiation of B cells occurs in the B cell follicle. B-1a, B-1b and B10 populations are still very poorly characterized.

In mice the presence of the surface marker CD5 distinguishes a B cell population with distinct characteristics, called B-1 cells. They develop early in ontogeny, they tend not to undergo somatic hypermutation (SHM), and they secrete IgM antibody with polyspecificity, including binding to self-antigens (Dorshkind and Montecino-Rodriguez, 2007). B-1 cells are so far the best characterized in mice. B cells expressing CD5 were also found in humans , and at least a subset of these cells might have similarities to those of murine B1 cells. Still, phenotypically and functionally distinct sublineages are not well described. Murine B-1a and B-1b cells seed the peritoneal and pleural cavities, however their origin and their precursors have been controversial. The pleuroperitoneal milieu appears to influence the functional characteristics of both B-1a and B-1b B cells as well as of the relatively small proportion of B-2 cells that reside in these sites (Berberich et al., 2007; Hastings et al., 2006). LPS from commensal bacteria presented by DCs can induce both the proliferation of B-1 B cells as well as their differentiation into IgM-secreting short-lived plasma cells. Antigen-specific B-1 B cells can be induced to switch in a T-cell-independent manner into IgA-secreting cells (Fagarasan and Honjo, 2003).

The $CD5^-$ B cell population is called B2 or conventional B cells, which include a number of subpopulations that represent different maturation stages. Depending on surface markers and the immunoglobulin isotype expressed conventional mature B cells can be distinguished as naive B cells ($IgD^+IgM^+CD27^+$), GC B cells (Fas^+GL7^+), switched memory B cells ($IgM^-IgD^-CD27^+$), and plasmablast ($CD38^{high}$, IgM^-) (Allman and Pillai, 2008; Chung et al., 2003).

Additionally, B cells with regulatory functions have been described (Mizoguchi and Bhan, 2006) and one phenotypically distinct subset, called B10 B cells, has been shown to regulate T cell-mediated inflammatory responses by means of IL-10 production (Yanaba et al., 2008).

1.2.2 Isotype switching and affinity maturation

The genes encoding immunoglobulins are assembled from 4 heavy chain segments (VH, D, JH and CH) and 3 light chain segments (VL, JL, and CL). There are 9 different heavy chain types (μ, δ, γ1-4, α1, α2 and ε), determining the isotype of the immunoglobulin and 2 light chain types (κ and λ). The gene encoding the μ constant region lies closest to the JH gene segment and therefore closest to the assembled V-region exon after DNA rearrangement which makes IgM the first immunoglobulin isotype to be expressed during B-cell development. The gene encoding μ is followed by the gene encoding the δ constant region, consequently the next immunoglobulin to be expressed in B cell development is IgD.

IgM and IgD are expressed by alternative splicing of the same VHDHJH exon to the μ and δ heavy chain exons (Fig. 2 upper panel). Th cells are involved in the maturation process of B cells outside the bone marrow. T cell-derived cytokines induce isotype switching, which is a process of DNA rearrangement. Switching moves the rearranged VHDHJH exon into a position upstream, bringing alternative exons of the C region in close proximity of the V region (Fig. 2, lower panel). This permits a functionally rearranged VHDHJH exon to be used to produce antibodies of different isotypes but the same antigenic specificity (Malisan et al., 1996). T cell–derived IL-4 causes switching to IgG1. IL-5 and TGF-β cause switching to IgA. IFN-γ appears to induce switching to IgG2 (Chaudhuri and Alt, 2004). Simultaneously, as B cells undergo isotype switching, an active process produces mutations, apparently randomly, in the antigen-binding portions of the heavy and light chains. If these mutations result in loss of affinity for the antigen, the cell loses access to important growth signals and dies. However, if the mutations result in increased affinity for the antigen the cell producing that antibody will have a proliferative advantage in response to antigen and grows to dominate the pool of responding cells. Somatic mutation and clonal expansion of mutated cells occurs in the GCs of SLOs (Schmidlin et al., 2009).

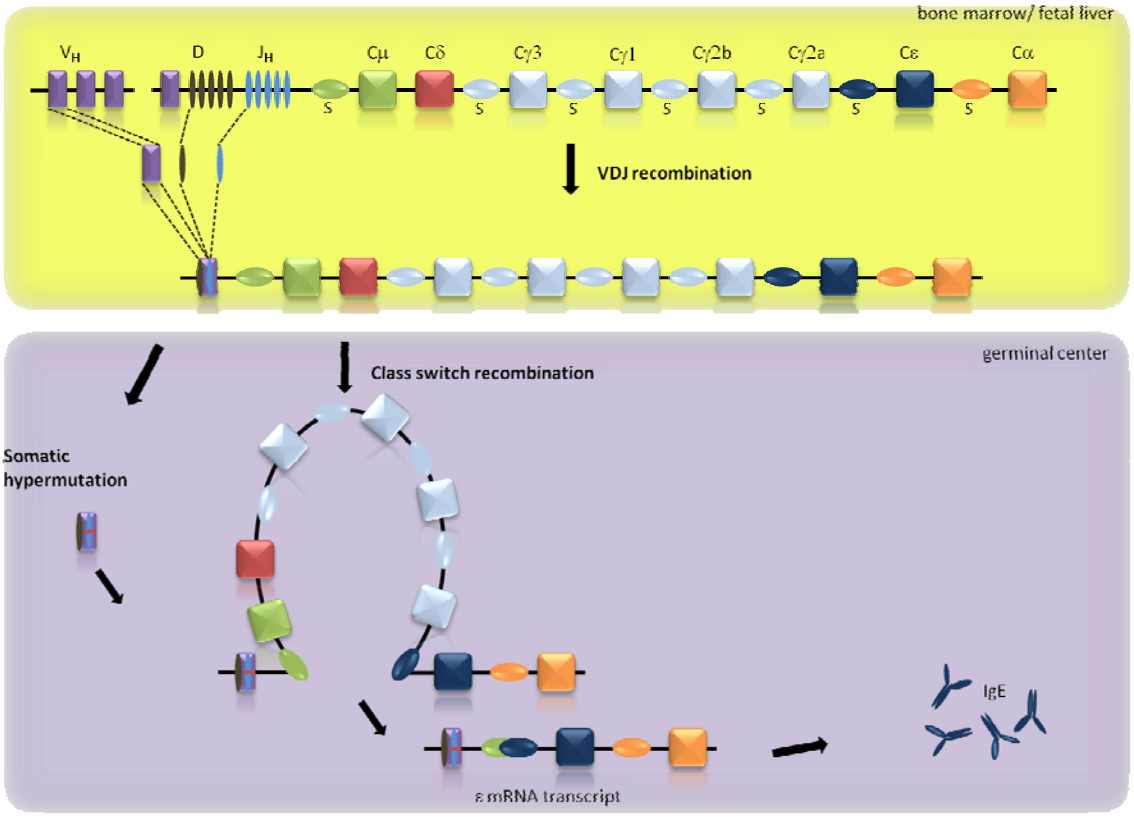

Figure 2. Rearrangement of the immunoglobulin heavy chain.. V(D)J recombination takes place in the bone marrow, while somatic hypermutation and CSR occur in the peripheral lymphoid tissues. V(D)J recombination selects one segment for each of the V, D and J segments from a pool of gene fragments and combines them into a variable (V)-region exon. Somatic hypermutation introduces mutations in the rearranged V exon., CSR brings the downstream constant (C) region exon in the proximity of the V exonen abling the production of antibodies with different isotypes.

1.2.3 Structure and diversity of antibodies

All antibodies share the same basic structure but exhibit high variability regarding the antigen binding region. The heterodimeric structure of an antibody is composed of two heavy (H) and two light (L) chains that are covalently connected via disulfide bonds. Each heavy and light chain consists of an amino terminal variable (V) and a carboxy terminal constant region (C). As mentioned above, the constant region of an antibodies H chain assigns the molecule its Ig class (IgM, IgD, IgA, IgG and IgE) conferring different effector functions, such as complement activation and mediation of cell cytotoxicity, depending on the isotype. On the other hand, the variable region of both, heavy and light chain, defines the antigen-specificity of the antibody and thereby accounts for the recognition of antigens. In any given antibody the two heavy and light chains are always identical, resulting in an antibody molecule with two identical binding sites and by that the ability to bind simultaneously two identical structures (Fig. 3). Proteolytic enzymes have been used to dissect the structure and function of the antibody molecule. Digestion with the protease

papain cleaves antibodies into three fragments. Two of them are identical and are called Fab fragments (fragment antigen binding). The Fab fragments correspond to the two identical arms of the antibody molecule, consisting of the light chain and the variable region together with one domain of the constant region from the heavy chain (Fig. 3). The third fragment is called Fc fragment and contains the two other domains from the constant region. As already mentioned, the Fc fragment is the part of the antibody able to interact with other molecules and cell receptors and therefore responsible of antibody effector functions.

The variable regions of H and L chain consist of three highly divergent segments called hypervariable regions that are flanked by conserved framework regions (FR). In three-dimensional space, the three hypervariable segments of the (VH) chain and the three hypervariable segments of the (VL) chain are brought together to form the antigen binding surface. The antigen binding surface is complementary to the three-dimensional structure of bound antigen, therefore the hypervariable segments are also referred to as complementarity-determining regions (CDR) (Janeway and Travers, 2001).

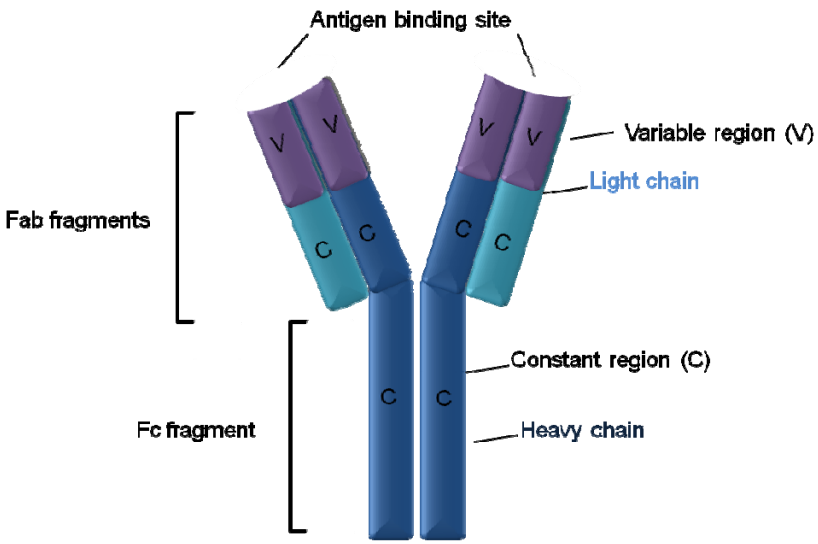

Figure 3. Structure of an IgG molecule. The protease papain cleaves immunoglobulins into three segments: Two Fab fragments which consist of the light chains (light blue) and a part of the heavy chain (dark blue) and the Fc fragment which consists of a part of the heavy chains. The Fab fragments contain the antigen binding site, the Fc fragment the effector region.

The enormous diversity of the antibody repertoire is generated by somatic recombination - also referred to as VDJ recombination - of Ig genes during the development of B lymphocytes in the bone marrow (Figure 2 upper panel). Three separate loci encode the two Ig light chains and the Ig heavy chain. Each light chain locus is composed of three different clusters of gene segments, referred to as variable (V), constant (C) and joining (J) gene segments. The IgH locus bears an additional cluster of diversity (D) gene segment situated between V and J clusters. The genes within a cluster are each separated from another by regions of non-coding DNA that vary in length. The somatic recombination of gene segments within each Ig

locus is a requisite step for the production of a functional antibody molecule and follows a precise order. The first recombination, occurring in the IgH locus results in joining of one of the D to one of the J gene segments. Thereafter, one of the V gene segments is joined to the DJ complex. Due to the lack of D gene segments within the light chain loci, somatic recombination directly joins one of the V to one of the J gene segments. The somatic recombination of gene segments within each locus occurs randomly. Therefore, the diversity that can be generated at each locus depends on the number of genes within its clusters. The diversity of antibodies is further enhanced by the so-called junctional diversity that is due to "non-precise" joining of gene segments. During somatic recombination, nucleases may remove nucleotides of the recombining gene segments. In addition, the enzyme terminal deoxyribonucleotidyl transferase (TdT) mediates the random addition of up to 20 non-germline encoded nucleotides at the junctions (Krangel, 2003). Taken together, theoretically the potential murine antibody repertoire comprises 10^{12}- 10^{14} different specificities. Because formation of the B lymphocyte repertoire in the bone marrow is antigen independent, it is also referred to as pre-immune repertoire.

1.3 T cells

T cells express an antigen-receptor, referred to as TCR that is related to immunoglobulins but strongly differs structurally from them. As all haematopoietic cells, T cells originate in the bone marrow, but develop in the thymus. In the thymus they recombine the TCR segments, of which there are four: α,β,γ and δ. Recombination begins at the γ, δ and β loci, and if expression of the γδTCR is successful, commitment to the γδT-cell lineage results (Lauritsen et al., 2006). γδ T cells leave the thymus to populate the lymphoid tissue and epithelia. Alternatively, successful β loci recombination results in βTCR expression, which pairs with the surrogate α receptor (pre-Tα) and forms the pre-TCR. It follows the recombination of the α-loci which generates, if successful, the αβTCR. Those thymocytes express CD4 and CD8 co-receptor molecules and undergo clonal selection by binding to peptide-loaded MHC molecules (peptide-MHC) expressed on thymic cortical epithelia. The interaction of TCR complex with peptide-MHC complexes is restricted by the specificity of the TCR and T-cell coreceptor. CD4 restricts interaction to class II MHC and CD8 to class I MHC molecule. T cell clones that bind with sufficient affinity receive survival signals and get positively selected. Surviving cells then lose the CD4 or CD8 coreceptor not involved in MHC recognition. These single-positive cells migrate to the thymic medulla, and those that react too strong with self-antigens presented by medullary thymic epithelial cells (mTEC) and APCs are deleted by apoptosis mechanisms (negative selection) (Carpenter and Bosselut, 2010). The CD4-expressing T cells have been classically designated as the helper lineage of the T cells and the CD8-expressing T cells are the cytotoxic lineage. Both represent the two major lineages among mature T cells.

Mature T cells are activated on interaction of their TCRs with peptide-MHC complexes. CD8 T cells can interact with peptides (9-11 amino acids in length) on almost any cell expressing

MHC class I. These MHC class I - restricted peptides are usually produced from proteins translated within the cell encoded either in the host genome or by infecting viruses or other intracellular pathogens. In contrast, the TCRs of CD4 T cells engage peptides complexed to MHC class II. In contrast to MHC class I, which is constitutively expressed in all nucleated cells, MHC class II molecules are only present on the surface of APCs and their membrane levels are increased by innate immune stimuli, including ligands for TLRs. As a consequence of their activation, after antigen encounter, APCs start migrating from the skin and mucosal sites to nearby lymph nodes, where interaction with T cells will initiate a immune response. T-cell activation is initiated when the TCR and associated proteins recognize a peptide-MHC complex on an APC, leading to a rapid clustering of TCR-associated molecules at the interface between T cells and APCs and the formation of a immunological synapse (Dustin, 2009). At the T-cell side the synapse is formed around a central cluster of CD3 and TCR, which bind specifically to the peptide-MHC complex, as well as CD4/CD8 molecules, which stabilize this interaction. Binding to MHC/peptide on the APCs by TCRs and at the same time CD4/CD8 in the synapse brings the cytosolic domains of these molecules into proximity. As a result, the CD4- and CD8-associated Src family protein tyrosine kinase Lck is able to phosphorylate tyrosine residues contained in cytoplasmic immunoreceptor tyrosine-based activation motifs of the TCR-associated CD3 chains. This results in the start of a signaling cascade leading to the activation of the T cell (Dustin, 2009).

Elimination of intracellular pathogens and tumors relies on cell-mediated immune response. CD8 effector T cells are the cells pivotal in this response and their function is distinguished by antigen-specific cytotoxicity restricted by MHC class I. After activation, CD8 T cells produce cytotoxic proteins including perforin and granzymes and secrete them at the point of contact with the target cell, the immunological synapse, therefore resulting in specific killing of the target cell without bystander cell damage. Perforin is a membrane-disrupting protein that allows granzymes to enter the cell and induce apoptosis. In addition to cytolysis, CD8 effectors produce mainly IFN-γ and TNF, which are pro-inflammatory cytokines.

1.3.1 $CD4^+$ T cells

Many aspects of the adaptive immune response start with the recognition of foreign MHC class II complexes on the surface of APCs by CD4 T cells (Banchereau and Steinman, 1998).

Naive $CD4^+$ T cells can differentiate into at least five main functional subsets: T helper-1 (Th1), Th2, Th17, regulatory T cells (Treg) and the B follicle-residing follicular helper T cells (T_{FH}) (Fig.4). Moreover recent studies showed an IL-9 producing subset called Th9 cells (Veldhoen et al., 2008) and a IL-22 producing subset called Th22 was found in human skin samples (Duhen et al., 2009). In addition, other $CD4^+$ T cells may also contribute to the adaptive immune response namely, Tr1 and NKT cells.

The differentiation of naive $CD4^+$ T cells into effector cell lineages underlies successful adaptive immune responses aimed at distinct categories of pathogens. Their functional specialization is coordinated by genetic programs that use different transcription factors to

direct expression of distinct soluble mediators and surface molecules that support interactions with other immune cells. The first paradigm for this functional diversification was the description of Th1 and Th2 $CD4^+$ effector subsets by Mosmann and Coffman in 1986 (Mosmann et al., 1986). Th1 cells were thought to be responsible for delayed-type hypersensitivity, activating macrophages through release of interferon (IFN)-γ and enabling them to kill intracellular pathogens. Th2 cells were considered the classical helper T cells providing help to B cells to generate class-switched antibodies. GATA-3 was identified as the transcription factor for the Th2 lineage, while for the Th1 lineage Tbet was described as the key transcription factor (Murphy et al., 2000). In Th2 cells, the transcriptional activation of GATA-3 provides a self-reinforcing feedback circuit (Ouyang et al., 2000). Likewise, T-bet induces its own expression, either directly (Mullen et al., 2001) or indirectly (Afkarian et al., 2002). Furthermore the most characteristic cytokines those subsets produce, further the transcription of those factors, while inhibiting the expression of other transcription factors.

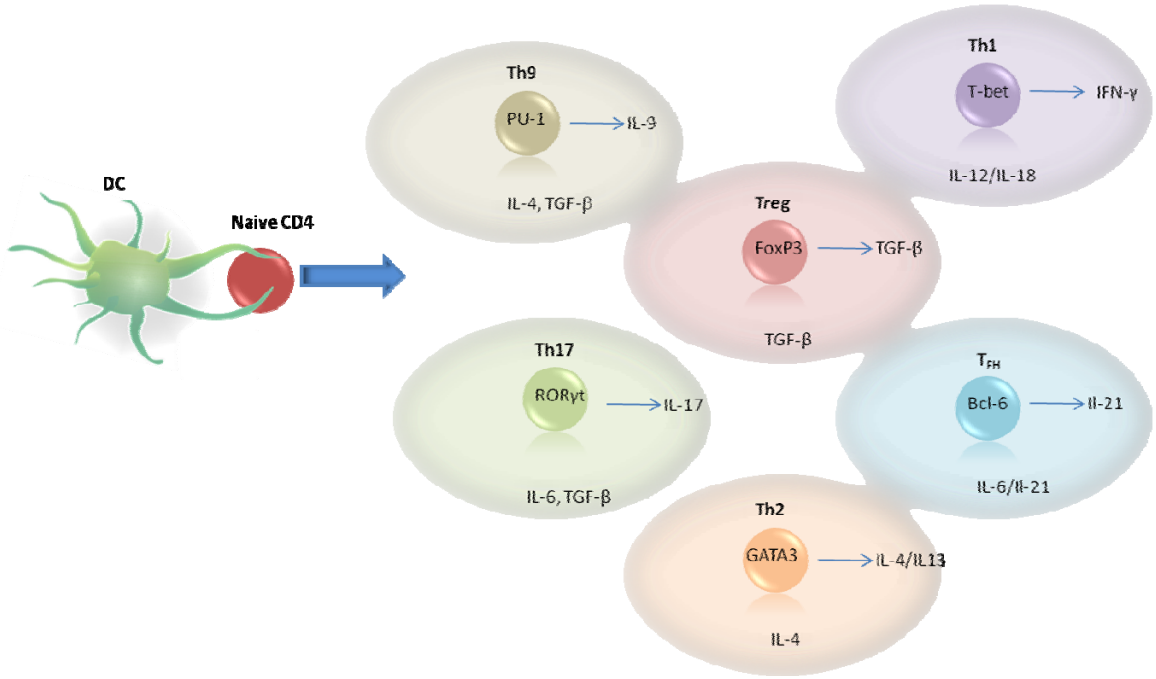

Figure 4. CD4 T cell lineages. Classical view of lineages and master regulators. These subsets express lineage defined transcription factors and produce selective cytokines. Environmental factors will support the development into the different lineages.

In 2003 the requirement for IL-23 in IL-17-producing $CD4^+$ T cells was recognized and with that, the classical Th1/Th2 model had to be revised. IL-17-producing cells, rather than Th1 cells, were established to play an important role in the animal model of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) (Murphy et al., 2003). Initially presumed to diverge from a common Th1 precursor (Bettelli and Kuchroo, 2005), the IL-17-producing cells, named Th17, were classified as a new subset on the basis of being

independent of the transcription factors GATA-3 and T-bet (Harrington et al., 2005; Park et al., 2005). The robust inducing conditions of IL-6 and TGF-β (Veldhoen et al., 2006) and the identification of RORγt and RORα as lineage-defining transcription factors (Ivanov et al., 2006; Yang et al., 2008) provided definitive support of Th17 as a separate subset.

Another main subset of $CD4^+$ T cells are Treg cells (Josefowicz and Rudensky, 2009), characterized by expression of the transcription factor forkhead box protein 3 (Foxp3). Treg cells derived from the thymus are thought to be a stable subset. However, Treg cells can be induced in the periphery from naive $CD4^+$ T cells by activation in the presence of TGF-β. Like the natural Treg cells (nTreg), induced Treg (iTreg) cells express Foxp3, but may be less stable and share circuitry with Th17 cells, which also require TGF-β for their differentiation (Curotto de Lafaille and Lafaille, 2009; Komatsu et al., 2009).

T_{FH} cells are localized in B cell follicles and essential for the generation of high-affinity and isotype switched antibodies and B cell memory (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000; Vinuesa et al., 2005). Although all activated $CD4^+$ T cells can migrate to follicular regions, T_{FH} cells preferentially reside there by virtue of their continuous expression of the chemokine receptor CXCR5. T_{FH} cells have the potential to secrete Th1, Th2 or Th17 cytokines (Crotty, 2011) and produce large amounts of IL-21, which acts in an autocrine manner together with IL-6 on their differentiation and expansion. The differentiation of T_{FH} cells depends also largely on the transcription factor Bcl-6, which is the key regulator of the T_{FH} lineage (Vogelzang et al., 2008; Yu et al., 2009b).

The $CD4^+$ T cell subpopulation are defined according to the lineage-indicating cytokine profile, their function and associated key transcription factors. Besides the aforementioned established $CD4^+$ T cell lineages there are various recent reports about additional new subsets.

A population of IL-9-producing cells, derived from Th2 cells by treatment with TGF-β, has been described (Veldhoen et al., 2008). IL-9 was once considered a Th2 cytokine but is now recognized as not being expressed together with IL-4, IL-5 or IL-13. Although suggested to be produced by Th17 or iTreg cells (Elyaman et al., 2009; Lu et al., 2006; Nowak et al., 2009), IL-9 is not expressed together with IL-17 or IL-22 and is not expressed by nTreg cells (Veldhoen et al., 2008). As this population has only been examined *in vitro*, it is unclear whether IL-9 producers should be considered a new subset, to be called Th9, or whether expression of this cytokine reflects adaptation of Th2 cells to a change in the microenvironment in the course of a response triggered by a pathogen or allergen. Recently the transcription factor PU-1 was identified as to be important for Th9 differentiation (Chang et al., 2010).

In another recent work, human, but not mouse, Th22 T cells (expressing IL-22 but not IL-17 or RORγt) were described (Duhen et al., 2009; Trifari et al., 2009) and may represent a skin-homing subset responsible for skin inflammation such as psoriasis. These cells preferentially develop when cultured with plasmacytoid DCs, which infiltrate psoriatic skin, but are independent of (and even inhibited by) IFN-α (Duhen et al., 2009), making their link to skin inflammation still uncertain.

1.4 $CD4^+$ T cell activation and costimulation

T cell activation begins with the binding of antigenic peptides to MHC molecule on the surface of an APC which is then, in its processed form, recognized by the TCR. The TCR has no intracellular signaling domain, but its membrane expression requires being physically associated to accessory molecules known as the CD3 complex (Salmond et al., 2009). The CD3 complex consists of three types of transmembrane molecules named CD3γ, CD3δ and CD3ε plus a mainly intracytoplasmic homodimer of 2 CD3ζ chains. TCR and CD3 complex together are termed the TCR complex.

The activation signal delivered by a ligand bound to the TCR complex, known as signal 1, is not sufficient by itself to completely activate a T cell. The TCR-peptide-MHC interaction is stabilized by the CD4 co-receptor. Besides the co-receptor a number of other costimulatory molecules are additionally required to enhance signal 1 and are referred to as signal 2. Costimulatory molecules on T cells (Fig. 5) can be divided, on the basis of their structural relationships, into three groups: immunoglobulin (Ig)-like receptors, integrins, and tumor necrosis factor receptor superfamily (TNFRSF) members. The majority of these molecules is expressed following TCR engagement and provides positive signals to the T cell encouraging maturation, proliferation, survival, and cytokine production.

Ig-like receptors, including the inducible costimulator (ICOS) as well as the constitutively expressed CD28, interact with ligands expressed on the surface of APCs. CD28 provides strong costimulation and, in almost all cases, is necessary for full T cell activation. Signaling through CD28 enhances protein tyrosine phosphorylation (Vandenberghe et al., 1992) and promotes cytoskeletal reorganization (Sedwick et al., 1999) and TCR association with lipid rafts (Viola et al., 1999). Furthermore CD28 signaling augments gene expression and stabilizes mRNA molecules from pro-inflammatory and survival genes (Cerdan et al., 1992; Wu et al., 2005). These signals continue until CD28 is displaced by cytotoxic T lymphocyte antigen-4 (CTLA-4), which arrests signaling and ends the activation process. Without the CD28 signal, T cells become anergic, which means they lose the ability to respond to antigen and acquire a passive, non-proliferative phenotype (Gimmi et al., 1993). This state of anergy has been claimed to be important to the development of peripheral tolerance ensuring that harmless antigens, encountered in the absence of other “danger” signals, do not trigger a potentially damaging autoimmune or allergic response (Gallucci and Matzinger, 2001). In contrast, T cells that receive full costimulation start proliferating, along with the production of cytokines, and ultimately survive and progress into the memory pool. The CD28 signal is so vital to full activation of naive T cells that it is known as signal 2 (Herrick and Bottomly, 2003).

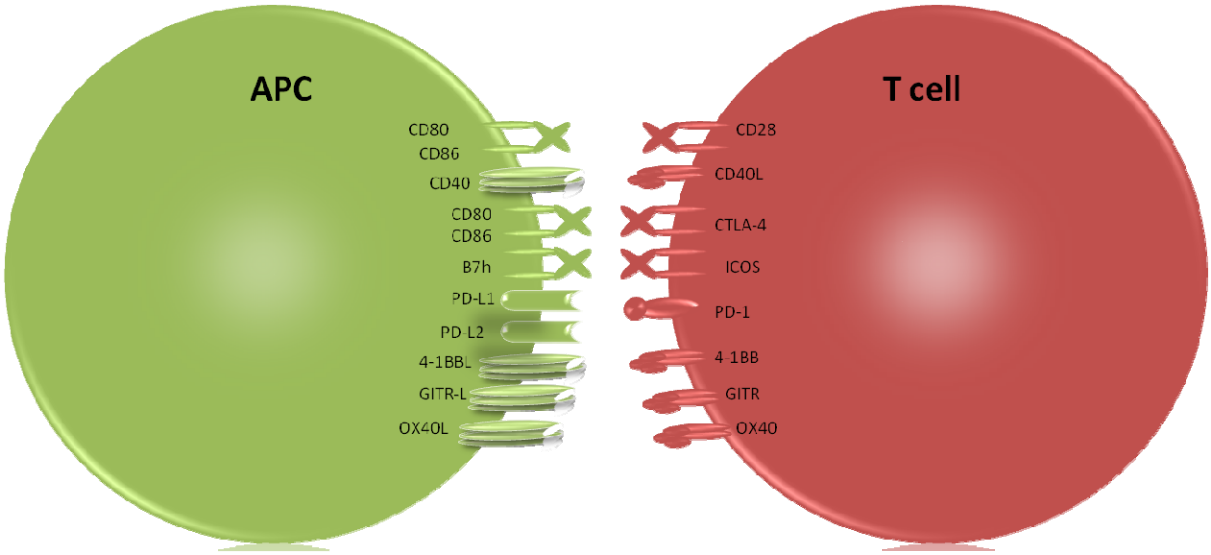

Figure 5. Costimulatory molecules. Costimulatory molecules can have either positive or negative stimulatory function. Depicted are some of the costimulatory receptors and their ligands expressed by APCs and T cells.

Integrins, such as leukocyte function-associated antigen-1 (LFA-1), also provide T cells with activation signals following interaction with their ligands on APCs, but are reliant on the presence of other costimulators, in particular CD28, to exert this effect (Dubey et al., 1995). They also play an important part in T cell migration, interacting with adhesion molecules on endothelial cells to allow T cell extravasation to the site of inflammation (Mentzer et al., 1986).

The TNFRSF includes a number of late costimulatory molecules, like OX40 and 4-1BB . Characteristically, these molecules are not constitutive expressed and provide positive signals to T cells inducing their survival and proliferation. In contrast to constitutive molecules, such as CD28, and early costimulators, such as ICOS, the TNFRSF members are mostly expressed on fully activated inflammatory T cells (Croft, 2003).

1.4.1 OX40

The OX40 molecule (also known as CD134) has a molecular weight of 50 kDa and is a glycosylated protein with three extracellular cysteine rich domains that act with OX40L on APCs. OX40 is a member of the TNFR superfamily and is expressed by T cells after activation (Takasawa et al., 2001). Both molecules, OX40 and its ligand, are membrane bound and trimerization, a common feature of TNF/TNFR superfamily members, is required for signaling (Compaan and Hymowitz, 2006). After ligation with OX40L, OX40 transmit signals to the cytoplasm through TNFR-associated factor (TRAF) molecules 2 and 5, which will activate NFκB-inducing kinase (NIK) and IκB kinases α and β which, in turn, phosphorylate and

degrade IκBα allowing its dissociation from NFκB. This results in the activation and translocation of transcripton factors like NFκB to the nucleus. Following this signaling cascade is the activation of the transcription of anti-apoptotic factors, such as Bcl-2 and BcL-X_L (Rogers et al., 2001), and the increase in expression of cytokine receptors such as the IL-12 receptor (Ruby et al., 2008).

Furthermore, up-regulation of CD25, the IL-2 receptor's α chain (Redmond et al., 2007), increases IL-2 signaling. OX40 signals in this fashion augmenting T cell survival and may also down-regulate the expression of pro-apoptotic molecules, like Bad and Bim, via phosphatidylinositol-3 kinase, further ensuring T cell survival and progression into the memory pool (del Peso et al., 1997; Rogers et al., 2001). The competitive recruitment of TRAF3 to the intracellular domain of OX40, arrests these signals by displacement of TRAF2 and prevents signaling to NIK (Takaori-Kondo et al., 2000). As well as its role in the activation and subsequent survival of naive T cells, OX40 provides several other signals that contribute to the inflammatory environment. Treg cells may express OX40 to a high level (Takeda et al., 2004), however, signaling through OX40 has been claimed to reduce the regulatory activity of Treg cells, abolishing their production of IL-10 (Ito et al., 2006) and reducing levels of the transcription factor Foxp3 (Vu et al., 2007). In addition, OX40 signaling breaks T cell anergy (Lathrop et al., 2004) and leads to activation of auto-reactive T cells that can then cause extensive tissue damage and potentially initiate autoimmunity.

In addition, it became clear that on OX40-OX40L interaction, signals are also transmitted to APCs via OX40L (Matsumura et al., 1999).

1.4.2 OX40L

OX40L (also called CD252) is part of the TNF superfamily and was first identified as glycoprotein 34 on human T-lymphotropic virus-I (HTLV-I)- transformed cells. It is a type II glycoprotein with a 23 amino acid cytoplasmatic tail and a 133 amino acid extracellular domain (Miura et al., 1991; Tanaka et al., 1985). Later it was found to be the ligand for OX40 (Baum et al., 1994; Godfrey et al., 1994). It is expressed as a trimer and has a TNF homology domain; thus, it is structurally similar to other molecules of the TNF superfamily. Although a number of the TNF family members can bind to several partners, until now there are no indications that OX40L can complex with anything other than OX40 (Croft, 2010).

OX40L which is expressed by various cell types, like airway smooth muscle cells, DCs and LTi, can be constitutively expressed or induced via TLR, as well as cytokine signaling (Burgess et al., 2004; Calderhead et al., 1993; Imura et al., 1996; Ohshima et al., 1997). The signaling pathways of OX40L are less well studied then those of OX40, but it is known, that c-fos and c-jun mRNA levels increase in endothelial cells following OX40L ligation (Matsumura et al., 1999). Additionally there are a number of functional outcomes of binding depending on the type of cell investigated. OX40L ligation on DCs leads to increased production of pro-inflammatory cytokines (Ohshima et al., 1997), B cells receive maturation signals for differentiation into plasma cells and increase Ig production (Stuber and Strober, 1996), and

endothelial cells start producing RANTES, a T cell-attracting chemokine, suggesting a role for OX40L signaling in T cell migration (Kotani et al., 2002). OX40 and OX40L show an aligned expression profile. Both molecules are upregulated after MHC/peptide-TCR interaction, reach their maximum expression 2–3 days later, and are are downregulated after 5-6 days (Calderhead et al., 1993; Gramaglia et al., 2000; Gramaglia et al., 1998).

1.5 T cell independent immune responses

Mature B and T cells continuously circulate through the body and the SLOs surveying the body for inflammatory signals and foreign antigens. Mature B cells within the SLOs express the chemokine receptor CXCR5, directing them to B cell follicles, whereas T cells express the chemokine receptor CCR7, directing them to the T cell area. After lymphocyte antigen encounter there are several pathways possible, depending on the type of antigen.

TI antigens can induce a humoral immune response without T cell help, and are characterized by the fact that they do not induce immunologic memory. TI antigens are generally divided into two categories, TI-1 and TI-2 antigens. TI-1 antigens, as for example LPS produced by gram negative bacteria, are potent B-cell mitogens, capable of inducing non-specific, polyclonal activation of B cells usually through TLRs (Richards et al., 2008). In contrast, TI-2 antigens can only activate mature B cells. The TI-2 antigens are macromolecules with repeated molecular patterns, such as polymerized proteins or polysaccharides, that are able to interact with several immunoglobulin receptors on the surface of a B cell and cross-link them. Such cross-linking can deliver a partially activating signal that can induce plasma cell development when additional signals are provided by cytokines or other cell contacts provided by DCs or macrophages (Vos et al., 2000). In many cases the antigens themselves might also provide more than one activating signal because some antigens also interact with other receptor systems, such as activation of the TLR9 pathway by CpG DNA (He et al., 2004). Transmembrane activator and CamL interactor (TACI), which is expressed on activated B cells represents another important signaling system in direct DC-B-cell interactions (Lee et al., 2008). One TACI ligand, a proliferation inducing ligand (APRIL), is expressed on a broad range of leukocytes. Another TACI ligand, B cell-activating factor (BAFF) is expressed on DCs and myeloid cells. The proteolytic cleaved soluble form of both ligands mediates the binding not only to TACI, but also to the other receptors of BAFF and APRIL (Lopez-Fraga et al., 2001; Schneider et al., 1999). In combination with the signals described above, binding of BAFF and APRIL to TACI can promote immunoglobulin isotype switching independently of T cells (He et al., 2004; Lee et al., 2008).

1.6 T cell dependent immune responses

T cell dependent (TD) antigens cannot induce a humoral immune response without T cell help, and they do induce immunologic B and T cell memory. After activation by a TD antigen B cells upregulate CCR7, which leads to the migration to boundary between B cell follicle and

the adjacent T cell area (Reif et al., 2002). Antigen activated B cells engage in cognate interactions with T cells specific for the same antigen (Garside et al., 1998). Activated B cell can then either become extrafollicular short-living plasmablasts or move into the B cell follicle to establish a GC. The mechanisms responsible for this fate decision remain poorly understood. Some studies suggest that the antigen affinity of the BCR, the level of BCR engagement, costimulatory signals received from T cells or all factors together could be involved in the fate decision process (Benson et al., 2007; Dal Porto et al., 1998; Paus et al., 2006; Shih et al., 2002). The migration of activated B cell to the distinct microenvironments and the subsequent differentiation into plasmablast or GC B cell is mediated by their differential expression of chemokine receptors. Short-lived plasmablasts downregulate CXCR5 and upregulate CXCR4 (Chan et al., 2009) and EBI2, whereas GC founder B cells retain the CXCR5 expression and downregulate EBI2 (Gatto et al., 2009; Pereira et al., 2009), causing them to slowly move toward the center of the follicle and to give rise to a GC.

As the immune response progresses, the extrafollicular plasma cells die within a few days, whereas long-lived plasma cells and memory B cells, created in the GC, begin to differentiate in the GC (Tarlinton and Smith, 2000). Many of the long-lived plasma cells migrate into the bone marrow, where they secrete antibodies for several weeks or longer (Moser et al., 2006).

1.6.1 Germinal center reaction

In 1884 Walther Flemming described areas with high mitotic activity in SLO. He named those structures germinal centers since he believed that they are the site of lymphocyte generation or germination (Flemming W., 1885). The term was kept, eventhough it is now understood that the site of lymphocyte origin is the bone marrow rather than the GCs. However, GCs are the main source for effector B cells and by that are indispensable for the humoral immune response against pathogens. More specifically, GCs produce antibodies with an increased affinity and allow immunoglobulin class-switch, resulting in a more specific response. Morover, they generate memory B cells, which are crucial to mount a secondary immune response.

Chance encounter of antigen-specific B cells with T cells at the T-B boundary is the first crucial step to activate B cell proliferation and to initiate the induction of the GC reaction. The interaction of CD40, which is constitutively expressed by B cells, with its ligand CD40L, expressed by activated T cells, is crucial during this encounter (Foy et al., 1994a; Han et al., 1995). In addition to expressing CD40L, activated T cells also secrete cytokines that deliver signals through specific cell-surface receptors that serve to drive B cell proliferation and differentiation. Thus T cells are essential for normal GC formation. Cytokine signals also play a central role in triggering CSR, one of the central events of the germinal center reaction (GCR). Signals delivered through cytokine receptors lead to the targeting of the inducible enzyme cytidine deaminase (AID) (Stavnezer et al., 2008).

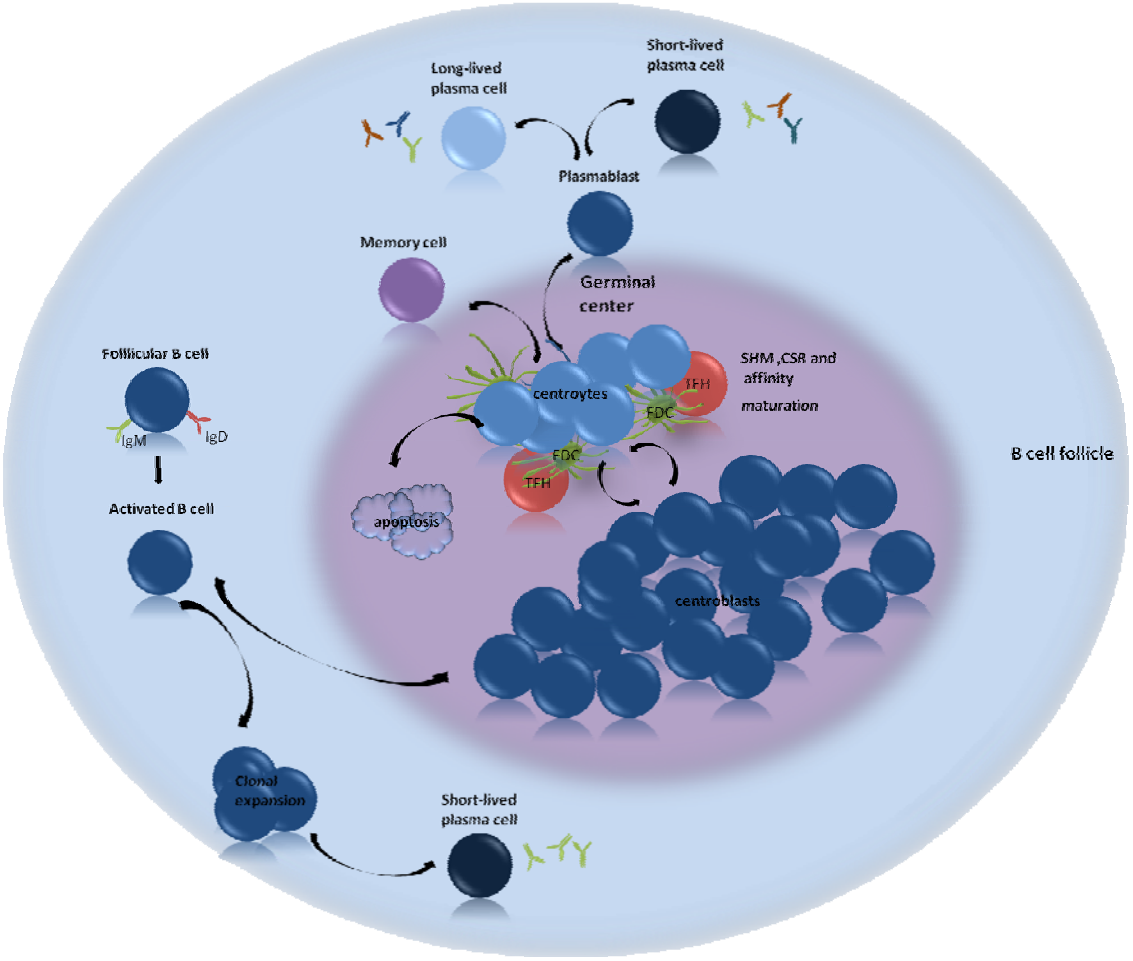

Figure 6. Schematic overview of the germinal center reaction. After activation B cells follow one of two fates: to become short lived plasma cells or to initiate a germinal center reaction.

In the classical view the GC initially only contains fast dividing centroblasts with downregulated surface immunoglobulin expression, but shortly after resolves into dark and light zone, which then also contain non-dividing centrocytes, that do express surface immunoglobulin (MacLennan, 1994; Nieuwenhuis and Opstelten, 1984). The dark zone is localized close to the T cell area and contains a high density of centroblasts. The light zone is the region of the GC with centrocytes at a low density and rich on FDC, which function to trap immune complexes, but also create a network for the immune cells to crawl on. The classical distinction into dark and light zone got lately challenged, as new techniques like 2-photon microscopy allowed to reanalyze this paradigm. Live-imaging studies demonstrated that B cells move bidirectionally between the two GC zones, instead of the unidirectional migration assumed in the classical dark/zone light zone model Moreover, GC B cells in the two zones seemed to be similar in size and morphology and showed comparable levels of DNA synthesis, challenging even more the traditional model, that describes centrocytes as small and nondividing and centroblasts as large and dividing (Allen et al., 2007b; Schwickert et al., 2007). However, studies in which migration between dark and light zone was blocked

failed to show an impact on GC size, cell division or the development of high-affinity antibodies (Allen et al., 2004; Nie et al., 2004). Finally the group from Nussenzweig used a photoactivatable fluorescent protein tracer to track GC B cells and showed, that B cell division is restricted to the dark zone and that B cells migrate undirectional from the dark zone to the light zone, supporting the original model. The decision to return to the dark zone and undergo clonal expansion seemed to be controlled by T_{FH} cells in the GC light zone, which differentiate between light zone and dark zone B cells based on the amount of antigen captured and presented (Victora et al., 2010).

Along with FDCs a second population of supporting cells can be found in the GC, the follicular helper T cells. They represent a minor population in the GC constituting only 5-20% of all GC cells. However, it was meanwhile established that they are crucial for the GCR, as mice that lack T_{FH} cells, but contain Th cells, are incapable of mounting a GC response (Yu et al., 2009b).

As Centroblasts proliferate in the GC, they acquire high rates of mutations in the variable regions of their immunoglobulin genes through the process of SHM. This process is associated with DNA strand breaks and introduces single nucleotide exchanges. As already for CSR the enzyme AID also plays here a central role. AID targets deoxycytidine bases in heavy and light chain IgV genes for demethylation, which are excised by uracil DNA glycosylase (UNG). Mutations are introduced by the filling of the resulting gaps by an error-prone polymerase and result in random changes in the affinity and specificity of the BCR. In the light zone clones with high affinity for the antigen are selected by their interaction with antigen captured by FDCs and receive survival signals whereas low affinity clones undergo apoptosis (Goodnow et al., 2010). The apoptotic cells are picked up by tingible body macrophages, which is a specialized subset of macrophages that resides in GCs (MacLennan, 1994). B cell clones positively selected within the GC differentiate either into long-lived plasma cells or memory B cells. Memory B cells have been shown to persist for a long period after antigen exposure and besides populating the splenic marginal zone they recirculate through SLO (Schittek and Rajewsky, 1990). GC-derived long-lived plasma cells migrate to the bone marrow and are a hallmark of long-term humoral memory and by that of successful vaccination (Manz et al., 1997).

1.6.2 Affinity maturation

A characteristic of humoral immune responses to TD antigens is the progressive increase of average antibody affinity (Eisen and Siskind, 1964). This so-called affinity maturation is now known to require further diversification of antigen activated B cells and is realized by introduction of somatic mutations at high rate (somatic hypermutation, SHM) into the sequences encoding the variable regions of the BCR. SHM was first experimentally validated by sequencing Ig genes of λ light chains of murine myeloma cells (Weigert et al., 1970). Subsequently, a selection process promotes those B cell clones expressing antibodies with higher affinity for the antigen (Berek et al., 1991; Jacob et al., 1991b).

Antigen activated GC precursor B cells migrate towards B cell follicles, settle down in the stromal environment created by FDCs and initiate an expansion phase characterized by brisk proliferation (Kosco et al., 1992; Tew et al., 1990). The vigorously proliferating centroblasts are submitted to SHM. Although the mechanisms underlying SHM were identified in the last years, both, the induction and regulation of SHM are still barely understood (Ganesh and Neuberger, 2011).

The rate of somatic mutation in Ig V-region genes is about 10^4-10^6 times higher than the spontaneous rate of mutation in other genes, a fact that somatic hypermutation owes its name. As a consequence of SHM, many of the GC B cells decrease their affinity antigen and some even loose Ig expression. However, some of the cells may acquire mutations that increase antibody affinity for the antigen. The next pivotal step of affinity maturation comprises the preferential selection and propagation of those GC B cells that have acquired a mutant BCR of higher affinity. Selection is attributed to the light zone of the GC that is rich in FDC and additionally harbors GC T cells. The centroblasts are believed to exit cell cycle and to migrate towards the light zone of GC. The non-proliferating now centrocytes compete for survival signals provided by engagement of their BCR with antigen on FDCs, unless they receive these survival signals, all centrocytes are prone to apoptosis. This disposition ensures that only the centrocytes that feature high affinity towards the antigen are retained. Surviving centrocytes are thought to internalize, process and afterwards present the antigen to T_{FH} cells, which in turn provide centrocytes with additional but essential survival signals. These signals may result in Ig class-switching and differentiation to long-lived plasma cells or memory B cells that subsequently exit GC.

1.6.3 Follicular helper T cells

The fact, that humoral response depends on help from cells of thymic origin, was one of the essential findings of the field of immunology. In these initial studies, cells from different sources were transferred to recipients that were immunocompromised by the lack of immune cells. Neither mice that only received cells from the bone marrow alone nor from the thymus were able to mount a full antibody response. However, when mice received cells from both origins they were able to respond in a normal way to the immunization (Claman et al., 1966; Miller and Mitchell, 1968). These interacting cells became later known as T and B cells and it is now know that the cognate help that B cells receive comes from a specialized subset of $CD4^+$ T cells called T_{FH} cells. These cells are a non-Th1/Th2 $CD4^+$ T cell population that expresses high levels of CXCR5 and show a potent ability to stimulate antibody production in B cells (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000).

The expression of the chemokine receptor CXCR5 together with the decrease of CCR7 expression also supports one of the fundamental features of T_{FH} cells, their localization within the B cell follicle, allowing them to select B cells and support their differentiation into plasma and memory cells. The ability of T_{FH} cells to provide help to GC B cells relies upon their high expression of costimulatory molecules like CD40L. CD40 signalling in B cells is

indispensable for their activation as well as differentiation, as CD40-knockout mice show complete lack of plasma cell development (Renshaw et al., 1994). ICOS, another costimulatory molecule that is highly expressed on T_{FH} cells is important for T_{FH} differentiation. However, whether ICOSL signaling is important for GC B cells remains controversial (Choi et al., 2011) and expression of ICOS is only a characteristic of human T_{FH} cells since in mice it is equally expressed by activated T cells (Deenick et al.; Yu et al., 2009b). In mice T_{FH} cells are furthermore characterized by the expression of the suppressive molecule programmed death-1 (PD-1) which is equally expressed in mice and humans. PD-1 expression on T_{FH} cells regulates survival and selection through engagement with PD-L2 or PD-L1 on GC B cells, preventing excess proliferation in a GC (Good-Jacobson et al., 2010). Furthermore, absence of PD1 is associated with alteration in the T_{FH} phenotype, as they produce less IL-4 and IL-21. As loss of PD1 signaling also leads to increased GC B cell apoptosis, PD-1 is thought to provide bidirectional signals (Good-Jacobson et al., 2010).

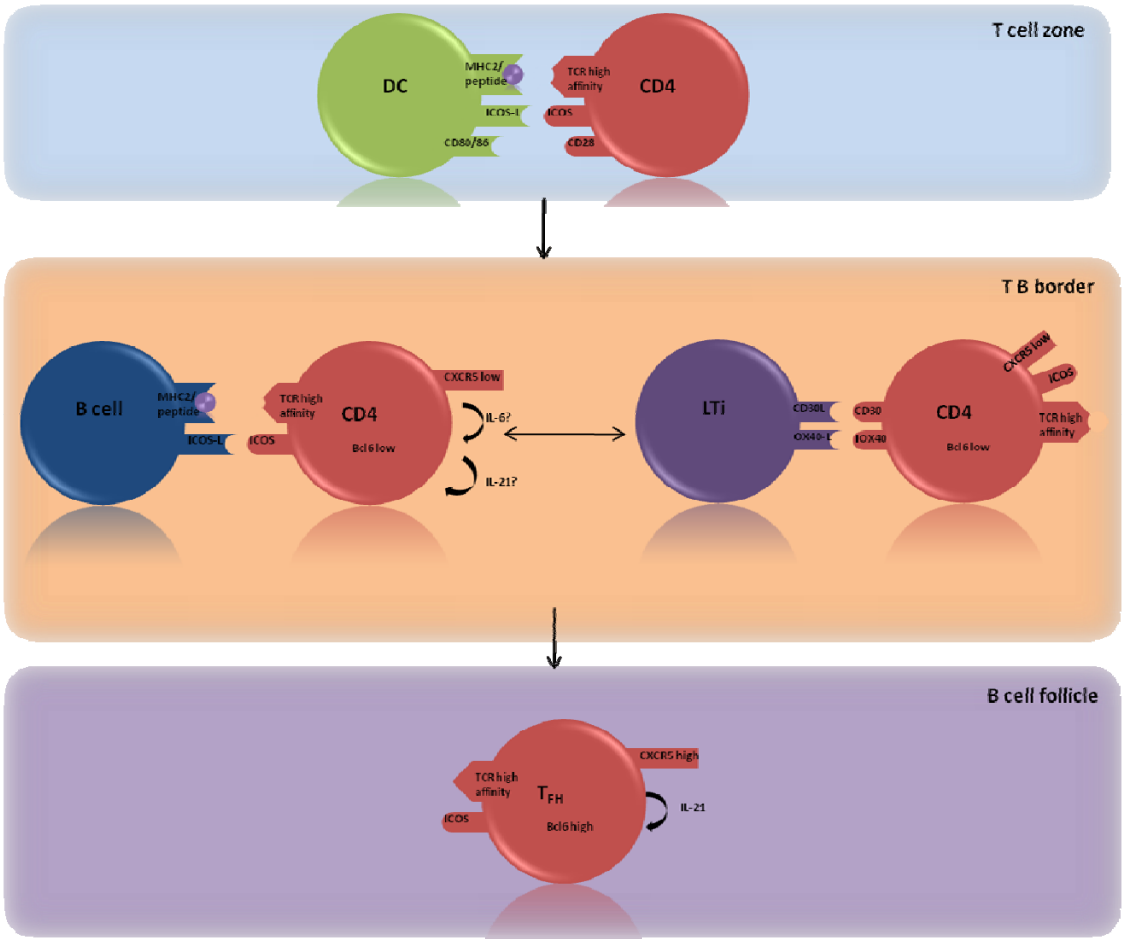

Figure 7. T_{FH} cell differentiation. T_{FH} differentiation is not yet fully understood. Depicted is a possible scenario for the development of a T_{FH} cell. Uncertain is still the role of IL-6 and IL-21 for T_{FH} differentiation or the putative interaction with LTi cells.

For several functional CD4 T cell subsets, specific transcription factors have been identified to act as master-regulators of subset-specific gene expression programs (Zhou et al., 2009). In 2009 three groups were able to convincingly show that Bcl6 is the master-regulator of the T_{FH} lineage, since overexpression of Bcl6 leads to augmented numbers of T_{FH} cells while knockout of bcl6 within the T cell lineage led to ablation of the T_{FH} cell population (Johnston et al., 2009; Nurieva and Dong, 2009; Yu et al., 2009b). Bcl6 is a transcriptional repressor originally identified as controlling GC B cell differentiation (Klein and Dalla-Favera, 2008). In T_{FH} cells Bcl6 controls genes different from those in GC B cells. The understanding of how Bcl6 controls T_{FH} cell differentiation is still limited, but it appears to alter microRNA expression and inhibit transcription factors involved in Th1, Th2 or Th17 differentiation (Yu et al., 2009b). In this regard, Bcl6 antagonism of Blimp-1 seems to be one of the main mechanisms by which Bcl6 blocks non- T_{FH} differentiation. Blimp-1 is known to direct effector cell differentiation in $CD8^+$ and $CD4^+$ T cells. The repression of Blimp-1 already skews the differentiation towards a T_{FH} phenotype (Johnston et al., 2009).

T_{FH} cells express the cytokine IL-21 at substantial levels. IL-21 is required for optimal GC formation, affinity maturation and proliferation of GC B cells (Linterman et al., 2010; Zotos et al., 2010). Moreover IL-21 induces Bcl6 expression in vitro and IL21 knockout mice have reduced numbers of T_{FH} cells. Therefore IL-21 is thought to be involved in T_{FH} cell differentiation (Fig. 7), even though this is still a controversial matter (Linterman et al., 2010; Nurieva et al., 2008; Poholek et al., 2010) .

Even though Bcl6 is inhibiting other $CD4^+$ T cell differentiation pathways, this blockade is incomplete, as T_{FH} cells are able to produce cytokines that are usually associated with other T helper lineages. Some T_{FH} cells express IL-4, which supports class-switch to IgG1 (Reinhardt et al., 2009), as well as GC B cell survival and selection (Cunningham et al., 2002; Ozaki et al., 2002). They are furthermore shown to express IFN-γ and IL-17, which promote class switch to IgG2a or spontaneous development of GCs and production of autoantibodies (Hsu et al., 2008; Reinhardt et al., 2009).

1.6.4 OX40L signaling and follicular helper T cells

T_{FH} cell differentiation depends on a variety of stimuli and is associated with the migration of the CD4 T cell into the B cell follicle. Those stimuli involve signals from DCs, $CD4^+CD3^-$ lymphoid tissue inducer (LTi) cells, B cells and follicular stromal cells (Linterman and Vinuesa, 2010). Antigen-presentation by DCs in the T-cell zone of the SLO and co-stimulation through CD28, consequently leads to upregulation of OX40 and CXCR5 expression by T cells (Walker et al., 1999). This initial upregulation of CXCR5 expression, in combination with downregulation of CCR7 expression, directs primed T cells to migrate to the boundary between T cell zone and B cell follicle (T-B boundary). In this location, they interact with B cells, but there is increasing evidence that they also interact with LTi cells (Ansel et al., 1999; Garside et al., 1998; Kim et al., 2003). LTi cells that reside in the T-B boundary express high levels of OX40L and CD30L and may provide survival signals to pre-T_{FH} cells, ensuring their

differentiation into T_{FH} cells (Fig.7) (Gaspal et al., 2005; Kim et al., 2003). Strikingly, combined deficiency of CD30 and OX40 did not affect primary immune responses, but had a strong impact on the generation of memory B cells (Gaspal et al., 2005). This is supported by the fact that OX40L signaling induces CXCR5 expression and influences the number of T_{FH} cells (Walker et al., 1999). However, the role of LTi cells is still poorly understood, as mice that overexpress OX40L at the surface of DCs have increased numbers of T cells in the follicles (Brocker et al., 1999) and i*n vitro* naive T cells that are stimulated by DCs overexpressing OX40L upregulate CXCR5 expression (Flynn et al., 1998). On the contrary, T cells from OX40 deficient mice are rendered unable to migrate into the follicles after immunization. Furthermore CD28 deficient mice that show a compromised OX40 function show the same phenotype (Fillatreau and Gray, 2003). To date it is not clear how OX40L signaling by LTi cells is involved in the development of T_{FH} cells, but there is surely strong evidence that LTi cells provide more than just tissue organization.

As a final remark about the importance of OX40L signaling for the development of T_{FH} cells, it has to be mentioned that IL-7 functions to some extent through the induction of OX40 expression and IL-7 secreted by murine lymphoid stromal cells was shown to be required for survival of memory T_{FH} cells that can induce secondary antibody responses (Gaspal et al., 2005).

1.6.5 Other Cells involved in the germinal center reaction

The density of B cells in the light zone is lower due to the presence of a specialized type of cells, FDCs. These are stromal cells in B-cell follicles typically displaying long dendrites and able to bind unprocessed antigen minutes after immunization and to retain it for months or years (Kosco-Vilbois, 2003). In fact FDCs are specialized in antigen capture and presentation of unprocessed antigen to GC B cells (Kosco-Vilbois, 2003). Both, primary and secondary FDCs present antigen in form of immune complexes, but whereas primary FDCs present antigen only on complement receptors, secondary FDCs in the GCs present antigen on complement and Fc receptors. The immune complexes consist of antigen and complement and/or immunoglobulin (Allen and Cyster, 2008). Small antigens are delivered directly via the fibroblastic reticular cell (FRC) conduits to the FDCs (Roozendaal et al., 2009). Bigger lymphborn antigens are transported by non-cognate B cells and subcapsular macrophages. Subcapsular macrophages first capture antigen (Junt et al., 2007) in form of immune complexes at the antigen entry, and these immune complexes are shuttled to the FDCs by non-cognate B cells through complement receptor binding (Phan et al., 2007).

FDCs can be distinguished as primary FDCs that reside in the follicle and secondary FDCs that are activated stromal cells localized in the GC (Allen and Cyster, 2008). Eventhough the exact ontogeny of FDCs is unclear, they are known to be of mesenchymal origin and unlike the name might suggest not related to DCs, which originate from hematopoietic precursors (Allen et al., 2007b; Endres et al., 1999). The formation of a secondary FDC network in the GC depends largely upon LT-α and TNF expression B cells. The stromal precursors of FDCs

receive signals through TNFR1 (Ansel et al., 2000; Endres et al., 1999; Matsumoto et al., 1997) and TNFR1 ligation leads to activation of NF-κB2 (Caamano et al., 1998)and RelB (Weih et al., 2001). However, TNFR1 signaling has no influence on the development of primary FDCs (Endres et al., 1999).

The FDC network is thought to be an important source of chemokines like CXCL13 (Cyster et al., 2000). Therefore FDCs play an important role in creating a specialized microenvironment that will attract cells expressing CXCR5 the chemokine receptor for CXCL13. CXCR5 is highly expressed by T_{FH} and GC B cells, which brings those cells into close contact, allowing cognate T cell-B cell interactions (Allen et al., 2004). Lack of CXCL13 leads to small, malformed GCs (Ansel et al., 2000).

Interaction of FDCs and GC B cells has been implicated in promoting survival of B cells and affinity maturation. FDC support survival by costimulatory signaling via CD21 (Roozendaal and Carroll, 2007) and CD40 (Gaspal et al., 2006), deliver integrin-mediated adhesion signals necessary for forming the B-cell synapse (Cannons et al., 2010), as well as signals to Notch receptors on B cells (Yoon et al., 2009). VCAM-1 and ICAM-1 on FDCs have furthermore importance on slowing down GC B cell migration facilitating their reception of survival signals (Koopman et al., 1994).

Besides their interaction with FDCs, GC B cells receive rescue signals from T cells and undergo either further rounds of proliferation or differentiate into plasma or memory B cells. Centrocytes that did not received enough survival signals undergo apoptosis. Those apoptotic cells are taken up by a specialized subset of macrophages called tingible body macrophages. These cells are characterized by nuclear debris in their cytoplasm, which represents to a large extent the degrading components of phagocytosed GC B cells (Vinuesa et al., 2009). Tingible body macrophages are not yet very well understood, but data from mice and humans indicate they play an important role in the progression of systemic lupus erythomatosis (SLE). SLE associated autoantigens can be found in apoptotic bodies and efficient removal seems to be essential to prevent autoimmunity (Casciola-Rosen et al., 1994). The protein milk fat globule-EGF factor 8 (MFGE8), expressed by tingible body macrophages, is fundamental for engulfment of apoptotic bodies through its binding to phophatidylserine, a molecule exposed on the surface of apoptotic cells. Mice deficient in MFGE8 developed manifestations associated with SLE (Kranich et al., 2008). Furthermore, a subgroup of patients with SLE showed loss of tingible body macrophages which was accompanied by accumulation of apoptotic cells in the GCs and apoptotic material at the surface of FDCs (Baumann et al., 2002). Taken together, although tingible body macrophages are yet poorly understood they seem to have an important role in the outcome of a GCR.

1.6.6 Germinal center regulation

Dysregulation of the mechanism controlling GC development and maintenance can lead to exaggerated or chronic GC reaction leading to immunopathology like autoimmunity or

cancer (Kuppers et al., 1999; Shlomchik et al., 1987). Therefore tight regulatory mechanisms have to be in place to control magnitude, as well as duration of a GCR.

GC formation and termination is regulated by a series of variables. Already the type of immunizing antigen has a great influence on the duration of GCs. Studies using haptenated protein for immunization showed that GC arise already at day 4 after primary immunization and the GCR has a duration of about 3 weeks (Jacob and Kelsoe, 1992), whereas studies using viral particles showed a GCR persisting for up to 3 months, driven by antigen depots in form of immune complexes. However, GCs observed in those experiments stop after 4 weeks to contribute to plasma cell numbers and antibody titers (Bachmann et al., 1996; Gatto et al., 2007). Therefore antigen persistence my play a role in triggering new GCs for a prolonged period of time or in regulating GC duration, but qualitatively those late GCs seem similar to GCs that are created in an environment that lacks T cells (de Vinuesa et al., 2000). Thus it remains to be shown the contribution of T_{FH} cells, as well as the degree of SHM and selection in those late GCs.

GC B cells are prone to apoptosis due to their proapoptotic program of gene expression characterized by low levels of Bcl-2 and Bcl-X_L and high levels of BIM and Fas. This is impressively supported by the fact, that GC B cells rapidly undergo apoptosis, if antigen encounter is not associated with survival signals (Klein et al., 2003; Liu et al., 1991a). This limits the lifespan of GC B cells under normal conditions and makes them dependent on survival signals. Defects in Fas in GC B cells have been shown to disrupt B cell homeostasis and can lead to autoimmunity (Hao et al., 2008). The process of SHM is associated with genetic instability due to fast proliferation and acquisition of mutations which can easily lead to a malignant transformation, if not carfully regulated. Therefore it is not surprising that the majority of B cell lymphomas originate from GC B cells, as proven by their expression of somatically mutated IgV gene segments (Kuppers et al., 1999).

Although it is well documented which factors can lead to GC dysregulation and by that to autoimmunity or cancer, it is to date still poorly understood what is the regulating mechanism that under normal conditions prevents those pathological outcomes.

Due to the lack of experimental data there are several mathematical models trying to explain GC regulation and by that also GC termination. One of the factors hypothesized is an increasing impairment of the engagement of B-cell receptors and antigen through either antigen consumption (Kesmir and De Boer, 1999) or antigen masking (Tarlinton and Smith, 2000). Both models assume that GC termination is regulated by the amount of antigen captured by FDCs and presented to GC B cells. Another approach presents processes related to T_{FH} cell proliferation as being the dominant step in the GCR (Moreira and Faro, 2006). This leads naturally to the hypothesis that regulatory mechanisms must exist affecting antigen-specific T_{FH} cells in order to prevent too intense GCRs and the production of autoantibodies.

1.7 Immune Tolerance

On a daily basis, each individual has to face thousands of pathogens, which are regularly eliminated. Part of the defense mechanism involved in this elimination is the generation of a broad repertoire of receptors by B and T cells to successfully recognize pathogens. As this is a random process, lymphocytes whose receptors are able to recognize self-antigens are sometimes generated, bearing the risk to lose tolerance to self-antigens, leading ultimately to autoimmunity. Therefore it is fundamental to ensure that cells bearing receptors with specificity for self-antigens are tightly controlled, either by eliminating them or preventing their activation, to ensure self-tolerance. According to their location lymphocyte tolerance mechanisms can be divided into two main categories, central and peripheral tolerance. Central tolerance involves mechanisms active during the maturation of T or B cells in the primary lymphoid organs, the thymus and the bone marrow. Peripheral tolerance concerns all mechanisms active after T and B cells exit the primary lymphoid organs. The key mechanism of central tolerance is clonal deletion, which is inactivation of self-reactive lymphocytes (Ohashi, 2003; Venanzi et al., 2004). However, central tolerance appears not to be 100% effective as all individuals harbor lymphocytes that can respond to self-antigens, underlining the importance of peripheral tolerance (Liblau et al., 1991; Sun et al., 1991). Thymic negative selection most effectively deletes those T cell precursors that express TCRs having high avidity for self-peptide-MHC complexes expressed on medullary DCs and medullary thymic epithelial cells (mTECs). Thus, autoreactive T cells escape negative selection in the thymus when their TCR is of sufficiently low avidity for self-peptide-MHC (Liu et al., 1995). These low-avidity autoreactive T cells together with T cells bearing TCRs being specific for tissue restricted antigens (TRAs) that are not expressed in sufficient amounts on mTECs have to be controlled by peripheral tolerance mechanisms. Tolerance mechanisms in the periphery involve clonal deletion, anergy, clonal ignorance, deviation, helplessness, as well as suppression (Walker and Abbas, 2002).

As peripheral tolerance mechanisms are crucial to maintain self-tolerance it is not surprising that peripheral tolerance and especially the activity of Treg cells, one of the key players of peripheral tolerance, became of great interest for scientists (Sakaguchi, 2004).

1.7.1 Ignorance mechanisms to maintain peripheral tolerance

One of the mechanisms of peripheral tolerance is the physical separation of self-reactive T cells from cells that express TRAs. Naive T cells migrate from the blood to the SLOs and, if no antigen is recognized, they recirculate through the efferent lymph back to the blood stream (Lammermann and Sixt, 2008). Naïve T cells express the chemokine receptor CCR7 and the homing receptor CD62L (also referred to as L-selectin). Naive $CCR7^{+}CD62L^{+}$ T cells migrate through the venule walls of lymph node post-capillary high endothelium guided by their homing receptors and concentration gradients of CCR7 ligands to enter the T cell-rich regions of the SLO. In the T cell area they interact with peptide-MHC complexes on DCs. (Banchereau and Steinman, 1998; Celli et al., 2007; Mempel et al., 2004). If the TCR does not

recognize any peptide presented by DCs, CCR7 becomes desensitized and recognition of efferent lymph sphingosine 1-phosphate (S1P) drives naive T cells out of the lymph node and back to the blood. Thus, under normal circumstances, naive T cells are excluded from nonlymphoid peripheral tissues, avoiding the possibility to come in contact with a tissue-resident cell expressing a high density of TRAs.

Unlike naive T cells, primed T cells follow an alternative circulation route with favored homing to local sites of inflammation (Lammermann and Sixt, 2008; Masopust et al., 2001; Reinhardt et al., 2001). The downmodulation of CCR7 and CD62L on these effector-memory T cells limits the migration into lymph nodes from blood through high endothelial venules. However, upregulation of P- and E-selectin ligands, as well as integrins, allows efficient migration from post-capillary venules into the interstitium of peripheral organs, predominantly under conditions of local inflammation or infection (Austrup et al., 1997; Masopust et al., 2004; Reinhardt et al., 2003). Subsequently, peptide-MHC recognition leading to effector-memory T cell differentiation increases to a great extent the risk of a potentially autoreactive T cells gaining access to tissues with high TRA expression. Although ignorance is an important strategy of peripheral tolerance the increased abundance of TRAs in certain tissues still challenge the immune system. Tolerogenic DCs, as well as Treg cells seem to provide the default mechanism to further ensure peripheral tolerance and use suppression of target cells as a central function.

1.7.2 Role of DCs in peripheral tolerance

Immature DCs are localized within peripheral tissues constantly scanning them for infection or injury (Banchereau and Steinman, 1998). In order to perform their function as antigen presenting cells, DCs constantly take up antigen through macropinocytosis or the mannose receptor to present internally processed antigens through MHC class II, or MHC class I complexes as a result of cross-presentation (Ackerman et al., 2006). In addition immature DCs express various C-type lectin receptors (for example mannose receptor) together with Fcγ and Fcε, and are capable of receptor-mediated phagocytosis. Activation of DCs through PRRs leads to downregulation of both mechanisms, downmodulation of antigen processing, expression of costimulatory molecules like CD80/CD86, as well as CCR7, which supports their migration to the SLOs (Bonasio and von Andrian, 2006). Due to their decreased processing activity, antigen captured at the time of DC activation is kept at the surface to be presented to T cells. Finally, full DC maturation is accompanied by synthesis of proinflammatory cytokines that amplify the immunogenicity of peptide-MHC complexes as well as regulate the differentiation of the responder T cells (Novak and Bieber, 2008). Additionally it has been also suggested that suboptimal antigen presentation leads to induction of Treg cells (Graca et al., 2005; Oliveira et al., 2011b) and that an incomplete form of DC maturation generates a tolerogenic DC. Incompletely matured lymph node DCs lead to unresponsiveness of antigen-specific TCR transgenic $CD4^+$ T cells (Hawiger et al., 2001). These results suggest that, in the absence of inflammation, lymph node and spleen resident DCs induce tolerance in

naive T cells that bear a TCR with high avidity for self peptide-MHC complexes presented by the DCs. Thus, polyclonal $CD4^+$ T cells expected to have high avidity to self peptide-MHC complexes can be controlled by peripheral tolerance mechanisms. Furthermore, certain dead or dying cells can also reinforce a tolerogenic DC phenotype (Liu et al., 2002). Apoptotic cells are not able to trigger DC maturation (Gallucci et al., 1999). TAM proteins which are universally expressed at the surface of apoptotic cells inhibit TLR signaling, as well as cytokine receptor mediated activation of NF-κB (Rothlin et al., 2007). In accordance with this observation mice lacking TAM receptors develop massive lymphoproliferation and systemic autoimmunity in association with hyperactivation of DCs (Lu and Lemke, 2001).

It is plausible to imagine a model where at the end of an immune response, decreasing levels of TLR ligands may be still sufficient to stimulate MHC class II and CCR7 upregulation, as well as migration of DCs to the draining lymph node, but the increasing uptake of apoptotic debris may also at the same time inhibit NF-κB dependent proinflammatory cytokine synthesis (Pasare and Medzhitov, 2004). The end result is a DC representing a tolerogenic phenotype. Self-peptide-MHC complexes on tolerogenic DCs eliminate potentially dangerous responder cells, while not affecting other naive T cells with potentially protective TCR specificities. Therefore, the recognition of a TRA on tolerogenic mature DCs by autoreactive T cells leads either to a functional inactivation or deletion of the T cell, thus preventing pathogenic recognition of self-peptide-MHC complexes within the peripheral tissues. Yet, tolerogenic DCs do not work alone to suppress T cell responsiveness.

1.7.3 Coinhibitory signals control peripheral tolerance

As already mentioned, costimulatory molecules give important activation, survival, proliferation or differentiation signals to T cells. However some of the costimulatory molecules are associated with suppressing T cell functions rather than having a stimulatory effect.

Clonal anergy of antigen-experienced T cells *in vitro* can be reversed by stimulation through CD28. CD28 signaling enhances the production of IL-2 and by that supports the IL-2R and mammalian target of rapamycin (mTOR) -dependent reversal of anergy (Colombetti et al., 2002; DeSilva et al., 1991; Harding et al., 1992; Mondino and Mueller, 2007; Powell et al., 1999). However the role of CD80 and CD86, the ligands of CD28 in the maintenance of peripheral tolerance is complex, as the outcome depends on the equilibrium between binding to CD28 or the structural homolog CTLA-4 (Finck et al., 1994; Miller et al., 1995; Racke et al., 1995; Salomon et al., 2000).

CTLA-4, which is expressed at later times after T cell activation, binds CD80 and CD86 with higher avidity than CD28 and antagonizes the functions of CD28 as it inhibits cell cycle progression (Krummel and Allison, 1996; Walunas et al., 1994). Animals deficient for CTLA-4 show spontaneous T cell lymphoproliferation and autoimmunity (Tivol et al., 1995; Waterhouse et al., 1995). Even though part of the counter-regulatory actions of CTLA-4 relates to its role in mediating the suppressive functions of $Foxp3^+$ $CD4^+$ Treg cells (Wing et

al., 2008), OVA-specific CTLA-4 deficient $CD4^+$ T cells transferred into mice lacking Treg cells and expressing OVA in the pancreatic β cells cause severe type 1 diabetes, whereas CTLA-4 sufficient $CD4^+$ T cells do not (Eggena et al., 2004), supporting a role for CTLA-4 independent of Treg cells

PD-1, another counter-regulatory molecule with some structural similarity to both CD28 and CTLA-4, has been implicated in the maintenance of peripheral tolerance based on the observation, that deletion of the gene encoding PD-1, or the genes encoding its two ligands PD ligand 1 (PD-L1) and PD-L2 leads to autoimmunity (Freeman et al., 2000; Keir et al., 2006; Nishimura et al., 1999).

Furthermore treatment with anti-PD-L1 mAbs was shown to reverse anergy and to even break already established tolerance in a type 1 diabetes model (Fife et al., 2006). PD-1 seems to play an important role in maintaining T cells anergic in part by counter-regulating migratory stop signals necessary for efficient TCR engagement, as treatment with anti-PD-L1 mAb, but not anti-CTLA-4 mAb, slows down the movements of anergic T cells within pancreatic islets (Fife et al., 2009).

In conclusion, CTLA-4/CD80, CD86 interactions seem to terminate proliferation and promote anergy induction during the primary response to self peptide-MHC complexes, whereas PD-1/PD-1 ligand interactions seem to control previously tolerized autoreactive T cells that enter the peripheral tissues and find self-peptide-MHC, maintaining them in an anergic state.

1.7.4 Blockade of costimulatory molecules as a therapeutic tool

Costimulation is one of the most intensively studied areas in current basic immunology. Blockade of costimulation has the advantage of selective inhibition of T-cell responses and brings the potential of inducing tolerance to specific antigens in both autoimmunity and transplantation (Judge et al., 1996; Lenschow et al., 1992; Webb et al., 1996). In transplantation, costimulation blockade represents a new generation of treatment using biologic agents that no longer need frequent monitoring of the patient nor implicate chronic toxicities(Vincenti, 2005), as costimulatory blockade was shown to induce infectious tolerance in association with activation induced cell death (Wells et al., 1999) and Treg cell induction(Graca et al., 2000)-. Examples of costimulatory molecules with therapeutic potential are glucocorticoid-induced TNF receptor (GITR), 4-1BB, ICOS, CD28 and OX40, as well as their ligands.

GITR deficient mice show reduced lung inflammation and pleurisy in a model of acute lung inflammation (Cuzzocrea et al., 2006) and blocking 4-1BB extends the survival of corneal allografts (Asai et al., 2007).

The importance of targeting 4-1BB in the context of cancer is supported by a study showing the restriction of 4-1BB expression to T cells found in hepatocellular carcinomas. 4-1BB expression could not be detected on T cells in the liver of healthy controls nor in peripheral blood of either group (Wan et al., 2004). Moreover in mice agonist signals through 4-1BB can

reduce the development of conjunctivitis by suppressing Th2 responses (Fukushima et al., 2005).

Blockade of ICOS seems to be another valid approach to use in therapy, as studies demonstrate increased expression of ICOS on $CD4^+$ T cells in the lamina propria of ulcerative colitis and Crohn's disease patients. These patients did not have elevated levels of ICOS in peripheral blood T cells and healthy people show low ICOS expression on T cells in both localizations. Furthermore, disease severity in inflammatory bowel disease positively correlates with the amount of ICOS expressed (Sato et al., 2004). In summary, these results indicate that costimulatory molecules in general provide a promising target for immune therapies. Especially because expression is restricted to effector T cells at the site of inflammation which limits dramatically the side effects, as it does not compromise systemically the immune system and does not reduce the memory and naïve T cell pool.

In the blockade of costimulatory molecules one has to distinguish between the blockade of early costimulatory signals like CD28 and blockade of late costimulatory signals like for example OX40.

In general, blocking late signals involved in T cell activation provides more specific protection than suppressing early ones, making late costimulatory molecules attractive targets for therapy. For instance, OX40L ligation on DCs induces their maturation (Ohshima et al., 1997), and another study suggests that an OX40L-induced enhanced maturation state in gut DCs may facilitate the development, activation, and proliferation of T cells in a mouse model of food allergy (Blazquez and Berin, 2008).

Yet, each costimulatory molecule has to be considered in its own, as their individual functions and relative expression kinetics will turn out to be pivotal. ICOS, for example, is expressed earlier than OX40 and, as such, it is also found that blockade of ICOS during flu infection does reduce inflammation, but also compromises viral clearance (Humphreys et al., 2003a).

Blockade of early costimulatory signals bears the risk of compromising immunity and the formation of T cell memory, but targeting late costimulatory molecules may not prevent pathology. Nevertheless, even though the complete set of effects of different costimulatory signals still have to be established, these therapies hold a lot of potential. Their use in the treatment of autoimmunity could provide similar benefits to other immunosuppressive treatments, such as anti-TNF therapy, with fewer associated side-effects. There seems to be for each pathology a certain timeframe that allows immune intervention by blockade of T cell costimulation. If such therapies are focused on those molecules activated at specific stages of T cell activation, it should be possible that the treatment allows preserving enough unaffected pathways to keep sufficient T cells to fight infection and create memory and still treating the particular pathology.

1.7.5 Blockade of OX40-OX40L signalling

The potential for an anti-OX40/OX40L therapy was first realized in a study that eliminated exclusively autoantigen-specific T cells by targeting OX40 in the context of autoimmune encephalomyelitis. In this approach autoreactive cells were eliminated leading to an amelioration of the disease without dampening the whole immune system (Weinberg et al., 1996). The unique upregulation of OX40 by effector cells within inflamed tissue, led scientists to explore the therapeutic potential of targeting OX40-OX40L interactions. Patients with multiple sclerosis show OX40 expressed on T cells that infiltrated the brain and central nervous system (Carboni et al., 2003). T cells in the synovial fluid from rheumatoid arthritis patients also show high expression of OX40, which cannot be observed in healthy patients nor in T cells isolated from peripheral blood (Giacomelli et al., 2001; Yoshioka et al., 2000). In the former described diseases, pathology is localized in a certain tissue and so is OX40 expression. Moreover, *in vivo* constitutive expression of OX40L by T cells leads to the spontaneous development of autoimmune like diseases, which can be ameliorated by OX40/OX40L blockade (Murata et al., 2002). On the other hand, long term graft survival of MHC-mismatched grafts was successfully established after OX40L blockade (Curry et al., 2004) and a study of xenogenic islet grafts in diabetic NOD mice showed that anti-OX40L blockade was more effective in promoting long term graft survival than blockade of CTLA-4 (Honkanen-Scott et al., 2008).

The effectiveness of anti-OX40/OX40L therapy can be attributed to the specific targeting of activated T cells, but also in part to the fact that potentially targeted cells like Treg cells are not being affected. Moreover, OX40L blockade is not only effective in the context of autoimmunity and graft acceptance. In fact, in a mouse model of thymic stromal lymphopoietin (TSLP)-mediated allergic inflammation blockade of OX40L effectively inhibited Th2-mediated inflammatory response (Seshasayee et al., 2007). However, as OX40 deficient mice have severely impaired memory responses due to impairment of primary clonal expansion, it raises the question, to what extent OX40L therapy will affect subsequent infection and by that lead to serious side effects (Gramaglia et al., 2000). In this respect it has been shown that blockade of OX40 eliminated weight loss and cachexia in a murine model of viral lung infection without compromising viral clearance (Humphreys et al., 2003b). Furthermore, another study suggests that CD30 (another TNFRF member) may compensate for the loss of OX40L signaling, as mice deficient for both molecules show lack of memory and antibody responses. It appears that in this study insufficient interaction of LTi cells (which express OX40L and CD30L) with T_{FH} cells leads to the observed phenotype (Gaspal et al., 2005).

The importance of targeting OX40/OX40L signaling is also emphasized by the fact that a neutralizing anti-OX40L mAb is currently in clinical trial for asthma therapy and depending on the success could be next tested for therapy on patients with acute coronary syndrome (Croft, 2010).

The benefit of therapy targeting OX40L signaling in systemic diseases, as it is the case for systemic lupus erythematosus or HIV has still to be validated, especially since those patients show a much more general T cell activation (Patschan et al., 2006; Yu et al., 2006)

Even though the full impact of OX40L blockade in disease treatment, as well as prevention, has still to be established, the available data give a promising future perspective. Therapies targeting OX40L interactions, maybe in combination with other costimulatory molecules, will hopefully improve the currently available treatments and diminish the associated side effects.

1.8 Regulatory cells

Since the discovery of $CD4^+CD25^+$ Treg cells, the concept of regulatory cells in general has increasingly gained acceptance by the scientific community and excitement about the possibilities of these cells for the treatment of diseases is growing progressively. Several investigations have established beyond doubt the crucial role of regulatory cells in various diseases and aspects of inflammation (Chen et al., 1994; Groux et al., 1997; Powrie et al., 1994). Subsets of regulatory T cells with various phenotypes and different suppression mechanisms include the naturally occurring, thymus-derived $CD4^+CD25^+Foxp3^+$ nTreg cells and the peripherally induced $CD4^+CD25^+$ $Foxp3^+$ iTreg cells, as well as the inducible IL-10 producing type 1 regulatory T cells (Tr1) (Akdis et al., 2004; Robinson et al., 2004). Additionally, subsets of $CD8^+$ T cells (Hu et al., 2004; Siegmund et al., 2009; Smith and Kumar, 2008), γδ T cells (Seo et al., 1999), $CD4^-CD8^-$ T cells (Strober et al., 1996), IL-10–producing B cells (Mauri et al., 2003), IL-10–producing NK cells (Deniz et al., 2008), IL-10–producing DCs (Akbari et al., 2001), $Foxp3^+$NKT cells (Monteiro et al., 2010) and macrophages displaying suppressive properties have been observed (Edwards et al., 2006) and might contribute to control peripheral tolerance (Akdis et al., 2004; Tang and Bluestone, 2008). The interaction of regulatory T cells was for a long time thought to be restricted to effector T cells, this model has now to be revised, as regulatory T cells were shown to interact directly with neutrophils (Richards et al., 2010), B cells (Meiler et al., 2008), NK cells (Zimmer et al., 2008), and NKT cells (Santodomingo-Garzon et al., 2009). Moreover, attempts to identify the mechanism of action of regulatory T cells led to a long list of functions and molecules involved in their suppressive actions. Depending on the nature of the host, the disease context, the involved agents and the tissue analyzed distinct effector functions may display a different level of importance and moreover different regulatory cells could be involved. In the context of therapy it might be necessary to focus on a "personalized" approach with regard to the regulatory cell, as well as the mechanism of action involved, rather than a universal treatment.

1.8.1 $CD4^+Foxp3^+$ regulatory T cells

Sixteen years ago Sakaguchi identified a $CD25^+$ subpopulation among CD4 T cells able to control peripheral tolerance. In this pioneer experiment he showed, that lack of those cells

in a transfer system leads to devastating autoimmune diseases (Sakaguchi et al., 1995). Although CD25 expression has been useful in defining the $CD4^+$ Treg cell population in naive individuals, it became soon clear, that accurate discrimination between Treg cells and recently activated nonregulatory T cells, which upregulate CD25, is almost impossible. Increased expression of CD25, as well as GITR and CTLA-4 on activated nonregulatory T cells also suggests that expression of these molecules does not functionally define the Treg cell population or at best defines them only partially (Fontenot and Rudensky, 2005). Furthermore some Treg cells were found to be CD25- (Graca et al., 2002).

The identification of mutations in the foxp3 gene as the cause for a autoimmune disease called IPEX in humans and the same fatal immune dysregulation in scurfy mice was a milestone in the field, as it allowed to identify exclusively Treg cells and led to further characterization of this lineage (Bailey et al., 2001; Brunkow et al., 2001; Chatila et al., 2000; Wildin et al., 2001). Short after birth patients with IPEX syndrome present type 1 diabetes, thyroiditis, psoriasis, elevated IgEs and food allergies, which prevent normal food intake, as well as additional autoimmune pathologies such as massive lymphoproliferation and severe infections (Gambineri et al., 2003). Similar symptoms are found in mutant scurfy mice, including severe dermatitis, aggressive lymphoproliferation resulting in enlargement of SLOs, lymphocytic infiltration of multiple organs, hypergammaglobulinemia and autoimmune hemolytic anemia (Godfrey et al., 1991).

Foxp3 was meanwhile determined as the master regulator of the Treg cell lineage required for $CD4^+$ T cells to acquire a regulatory phenotype (Fontenot et al., 2003; Hori et al., 2003). Foxp3 induction in developing thymocytes commits those cells to the Treg lineage rather than to any other CD4 T cell lineage described (Yagi et al., 2004). Furthermore induction of Foxp3 expression in the periphery or *in vitro* via the TCR together with TGFβ induces conversion to a regulatory phenotype(Chen et al., 2003; Cobbold et al., 2004; Laurence et al., 2007; Niedbala et al., 2007). However, some reports suggest that Foxp3 expression is crucial for stabilization of the regulatory phenotype in the periphery rather than for the induction in the thymus (Lin et al., 2007; Williams and Rudensky, 2007).

Due to the fact that Treg cells can develop in the thymus or can be induced in the periphery by means of extrinsic factors like TGFβ and IL-2 or signals delivered through the TCR (Chen et al., 2003; Kretschmer et al., 2005; Zheng et al., 2004), people generally distinguish between naturally occurring Treg cells and Treg cell induced in the periphery.

Within the CD4 T cell population $Foxp3^+$ Treg cells represent 5%-10% under steady state conditions. However, their percentage is locally increased in microenvironments like those of tumors and chronic infections or in the context of oral tolerance induction (Belkaid, 2008).

1.8.2 Mechanisms of $Foxp3^+CD4^+$ Treg cell function

$Foxp3^+CD4^+$Treg cells control various aspects of the immune response, as well as immune homeostasis. Despite an enormous effort invested in unraveling the mechanism of action of

Treg cells, it is still not fully understood. Depending on the environmental context, Treg cells have been reported to suppress effector cells in a contact dependent manner, for example via killing of target cells using granzyme and perforin, as well as CD39, CD73 and LAG-3 (Sakaguchi et al., 2009). Besides cell contact dependent mechanisms, Treg cells can also act on effector T cells by the secretion of soluble factors like IL-10, TGFβ and IL-35 or the consumption of important survival factors like IL-2 (Tang and Bluestone, 2008). Moreover, Treg cell function can be also elicited at the side of APCs, decreasing their levels of costimulation and antigen presentation (Shevach, 2009; Tang and Bluestone, 2008; Vignali et al., 2008). Using intravital two-photon microscopy it was also demonstrated that Treg cells inhibit stable contacts between antigen-activated T cells and DCs making a complete activation impossible (Tadokoro et al., 2006; Tang et al., 2006).

However, besides this big variety of suppressive mechanisms by Treg cells, scientists are trying to point out one core mechanism under the control of Foxp3, responsible for suppression in all conditions and valid in mouse as well as the human system.

As such a molecule would have to show a similar disastrous outcome as Foxp3 when malfunctioning, CTLA-4 seems to be a legitimate candidate, as mice deficient for CTLA-4 show exacerbated lymphoproliferation, T cell mediated autoimmune disorders, as well as IgE hyperproduction, similar to what is observed in Foxp3 knockout mice (Wing et al., 2008). More evidence comes from the fact, that Treg cells constitutively express high amounts of CTLA-4 in mice (Read et al., 2000; Salomon et al., 2000; Takahashi et al., 2000) and humans (Miyara et al., 2009) and that Foxp3 together with other transcription factors binds to the promoter region of *ctla4*. (Marson et al., 2007; Ono et al., 2007; Wu et al., 2006; Zheng et al., 2007). A key function of CTLA-4-dependent suppression may lie in the competition for CD80/CD86 binding on DCs, as CTLA-4 binds with higher avidity as CD28 and will deliver suppressive rather than costimulatory signals.

CTLA-4 ligation activates the indoleamine-2,3-dioxygenase (IDO) pathway in DCs, which will lead to the production of immunosuppressive kynurenin or to activation of the immune-regulating transcription factor Foxo3, which ultimately will suppress cytokine production by DCs (Dejean et al., 2009; Grohmann et al., 2002).

Collectively these findings support the concept that CTLA-4 expressed by $Foxp3^+$ Treg cells is essential for their function as regulators of immune homeostasis and self tolerance. Depending on the environmental context and the type of immune response, other suppressive mechanisms, may operate along with or subsequent to CTLA-4–dependent mechanisms. For instance, perforin- or granzyme-expressing $Foxp3^+$ Treg cells are rare under healthy conditions but increase dramatically in a tumor environment (Cao et al., 2007). IL10 producing Treg cells are enriched at environmental interfaces like lung and colon and IL-10 deficiency does not affect systemic immune homeostasis, but leads to mucosal inflammation, supporting the importance of IL-10 for intestinal immune regulation (Maynard et al., 2007; Rubtsov et al., 2008). Moreover, Treg cells can express helper lineage specific

transcription factors to acquire the ability to specifically control Th1, Th17 or Th2 responses (Koch et al., 2009; Zheng et al., 2009; Zhou et al., 2008).

Taken together, there is a certain likelihood that besides a key mechanism several modes of suppression act synergistically and in a complementary manner. Moreover each suppression mechanism may exert its functions under certain environmental condition and associated to a particular biological context.

1.9 Aims of this thesis

Previous studies from our group have shown that treatment of mice subjected to allergic airways disease using an anti-CD4mAb during the time of allergic sensitization reduced significantly disease pathology, namely airways eosinophilia, production of IL-4, IL-5 and IL-13 in the lung and antigen-specific IgE. Moreover, treatment with anti-CD4 mAb led to induction of immune tolerance to the allergen, as recall responses were prevented (Agua-Doce and Graca, 2011). CD4 is an early costimulatory molecule because CD4 signaling is needed at the time of antigen recognition; as such, it is expressed on all T helper cells. Therefore CD4 blockade has a strong impact on the immune system and does not only affect cells that are involved in the immune pathology. Late costimulatory molecules on the other hand are exclusively expressed on activated cells, as such, targeting late costimulatory molecules like OX40L brings the potential advantage, that only cells involved in the disease pathology are being targeted. Therefore the first aim of this thesis was to study if blockade of OX40L would prevent the pathology in a mouse model of allergic airways disease, and if it would lead to immune tolerance. Those studies are described in chapter 2.

In chapter 3, a new suppressive $Foxp3^+CD4^+$T cell subset, which we named follicular regulatory T ($T_F$reg) cells, is described. There it is shown that follicular $CD4^+$ T cells from mLN include a subpopulation of $Foxp3^+$ T cells. This chapter also describes the phenotypic characteristics of $T_F$reg cells that clearly distinguish them from both T_{FH} and Treg cells. Moreover, we present data indicating that those cells originate predominantly from non-follicular Treg cells.

As $T_F$reg cells are exclusive to B cell follicles, chapter 4 investigates the impact of those cells on the GCR. As non-follicular $Foxp3^+$ Treg cells are known to have a strong impact on immune regulation, it could be that they somehow regulate the GCR. We show in this chapter that this is the case, as $T_F$reg cells have substantial impact on the magnitude of the GCR as well as in antibody production.

2. The influence of OX40L blockade in a model of allergic airway disease

2.1 Background

Asthma, which has been increasing in prevalence over the last years, leads to significant morbidity and mortality. It is characterized by chronic inflammation of the airways associated with massive infiltration of the bronchial mucosa by lymphocytes, eosinophils and mast cells, as well as goblet cell hyperplasia and thickening of the submucosa (Beasley et al., 1989; McFadden and Gilbert, 1992). The cellular events lead to the physiological manifestations associated with this disease such as airflow obstruction and airway hyperactivity (AHR) (Holgate, 1997).

Even though the origin of asthma can include various factors, the genetic predisposition of allergen-induced IgE synthesis is strongly associated with disease development (Cockcroft et al., 1977; Sears et al., 1991). In allergic asthma, the inflammatory process is initiated due to exaggerated response to commonly inhaled allergens. Atopic individuals develop allergen-specific IgE antibodies after repeated exposure to the allergen. As mast cells get activated by those IgE antibodies, the next exposure to the allergen will initiate the release of inflammatory cytokines. The allergic response can be divided into two phases: the early response, which takes place within minutes and is characterized by mast cell degranulation which will ultimatively drive a increase in airway smooth muscle tone leading to airway narrowing (Pauwels, 1989); and the late response which happens hours after allergen exposure and is associated with migration of eosinophils, neutrophils and lymphocytes to the lung parenchyma and airway epithelium leading to airway narrowing (Bousquet et al., 1990; Pauwels, 1989).

CD4 T cells, in particular Th2 cells, play a pivotal role as orchestrators of the inflammatory response in the pathogenesis of asthma (Lemanske and Busse, 1997).

Allergic asthma is, as mentioned above, characterized by high levels of serum IgE, chronic airway inflammation with massive infiltration of eosinophils, increased mucus production in the bronchioles and airway AHR. These asthmatic reactions are caused by allergen-specific Th2 cells producing IL-4, IL-5 and IL-13 (Mazzarella et al., 2000; Robinson et al., 1992; Wills-Karp, 1999). IL-4 regulates the production of IgE antibodies (Kuhn et al., 1991), IL-5 the development, activation and recruitment of eosinophils (Lopez et al., 1988) and IL-13 mucus production and AHR, most likely by affecting directly airway smooth muscle cells (Wills-Karp et al., 1998).

In order to release cytokines, T cells have to be activated via the TCR/peptide ligation and receive additional signals through costimulatory molecules (Mueller et al., 1989). Some members of the TNF and TNFR superfamily are mediators of this T cell costimulation (DeBenedette et al., 1997; Grewal and Flavell, 1996; Smith et al., 1994). One such receptor-ligand pair, OX40 mainly expressed on activated $CD4^+$ T cells (Baum et al., 1994; Calderhead

et al., 1993) and OX40L expressed on activated B cells, DCs and endothelial cells (Godfrey et al., 1994; Ohshima et al., 1997), was shown to enhance T cell proliferation and cytokine production (Akiba et al., 1999; Baum et al., 1994; Calderhead et al., 1993).

It has been proposed that OX40/OX40L signaling may promote differentiation of Th0 cells into Th2 effector cells (Flynn et al., 1998; Ohshima et al., 1998). Furthermore, OX40/OX40L signaling seems to mediate adhesion of activated T cells to vascular endothelial cells, which could play an important role in the migration of T cells to the site of inflammation (Imura et al., 1996).

These observations suggest that OX40/OX40L interaction may be involved in the pathogenesis of allergic asthma by controlling the development of pathogenic Th2 cells and/or the migration of effector Th2 cells into the lung. Consistently, the development of allergic asthma is greatly dimished in OX40-deficient mice (Jember et al., 2001).

Furthermore it was shown that OX40L blockade could prevent allergic airway disease in rodents and non-human primates (Seshasayee et al., 2007). Results from our group showed that co-receptor blockade using anti-CD4 monoclonal antibody (mAb) induces tolerance to allergic agents (Agua-Doce and Graca, 2011). We therefore investigated the efficiency of OX40L blockade in preventing of disease in an animal model of allergic asthma and the potential of OX40L therapy in inducing tolerance to a model allergen.

2.2 Materials and Methods

Mice and immunization

Balb/c mice were bred and hosted in the specific pathogen free (SPF) facility at the Instituto Gulbenkian de Ciência. All mice were sex matched, kept under specific pathogen-free (SPF) conditions and used between 6-8 weeks of age. All animal experiments were conducted according to the IGC animal ethics committee under a license by Direcção Geral de Veterinaria (Portugal).

Sensitization

Unless otherwise stated in the text, animals were sensitized intraperitonally with 20 µg of Ovalbumin (OVA, Sigma, St Luis, USA) emulsified in 2 mg of endotoxin-free aluminum hydroxide (alum, Alu-gel-S, Serva, Heidelberg, Germany). OVA was previously run through a DetoxyGel column (Pierce, Rockford, USA).

Antibodies and reagents

Non-depleting anti-OX40L (OX89) was produced in our laboratory using Integra CL1000 flasks (IBS, Chur, Switzerland) and purified from culture supernatants by 50% ammonium sulfate precipitation and dialyzed against PBS. The purity was examined by native and SDS gel electrophoresis. The hybridoma cell lines were generously provided by Herman Waldmann (Oxford).

Bronchoalveolar Lavage (BAL)

The airways were washed three times through the trachea with 1 ml of cold PBS 10% BSA (Sigma). The collected BAL was centrifuged, resuspended in PBS and the cells counted with a hemocytometer. Samples were then cytospinned in a Cytospin2 (Shandon) for 5 min at 1000 rpm and dried over night, followed by Giemsa-Wright (Sigma) staining. For differential cell counts at least 200 cells from each sample were counted, using blinded slides, to determine the relative frequency of each cell type.

ELISA

Serum titers of total and OVA-specific Immunoglobulins were measured by ELISA using a IgG1 (SouthernBiotech, Birmingham, USA) and IgE Kit (BD-Pharmingen), with anti-OVA IgG1 standard from Serotec and anti-OVA IgE standard from Abcam (Cambridge, UK). Cytokine titers were determined in lung homogenates. Lung tissue was collected, homogenized at 100 mg/ml in PBS 2% BSA (Sigma), centrifuged at 3000 rpm for 10 min and the supernatant

collected. Cytokine ELISA were performed using the following kits: IL-10, IL-13, IFN-γ, TNF (Peprotech, London, UK), IL-4, IL-5 (BD-Pharmingen).

Histology

Lungs were perfused with 4% formalin solution (Sigma), collected and sectioned. Staining was performed using hematoxilin/eosin and mucus containing cells were revealed using a periodic acid-Schiff (PAS) stain. Photographs were taken with a Leica DM2500 microscope and a Leica DFC420 camera.

Respiratory mechanics and methacholine responsiveness

Airway responsiveness was determined 24 hours after last intranasal OVA challenge. Changes in the respiratory input impedance (Zrs) were measured using a modification of the low frequency forced-oscillation technique (LFOT) in mice anesthetized with 10 µl/g of xylazine (2 mg/ml, Ronpum, Bayer, Germany) and ketamine (40 mg/ml, Merial, Lyon, France), tracheostomized and ventilated (FlexiVent, SciReq, Montreal, Canada). Mice were hyperventilated at 450 breaths/min and Zrs was measured during periods of apnea using a 16 s signal containing 19 prime sinusoidal frequencies. Calculation of airway resistance (Raw) is obtained from the Zrs spectrum using FlexiVent software (SciReq). AHR was measured by exposure to an aerosol containing increasing doses of methacholin (MCh, Sigma), following a baseline measurement after the delivery of a saline aerosol.

2.3 Results

2.3.1 Anti-OX40L treatment prevents allergic AHR

To analyze the effect of OX40L blockade in allergic asthma we took advantage of a well established murine model of airway hyperreactivity induced with OVA. Three groups of Balb/c mice were first sensitized with 20 µg OVA-alum given twice i.p. with a 14 day interval, followed 7 days later by an intranasal challenge with 50 µg OVA in three consecutive days between day 21 and 23, to elicit the inflammation in the airways (Figure 1A). This protocol leads to inflammatory infiltrates in the airways, with eosinophils representing the majority of these cells (Figure 1B). However, when OX40-OX40L interaction was blocked, with a non-depleting monoclonal antibody against OX40L, the inflammatory infiltrates in the BAL, predominantly eosinophils, could be prevented (Figure 1B).

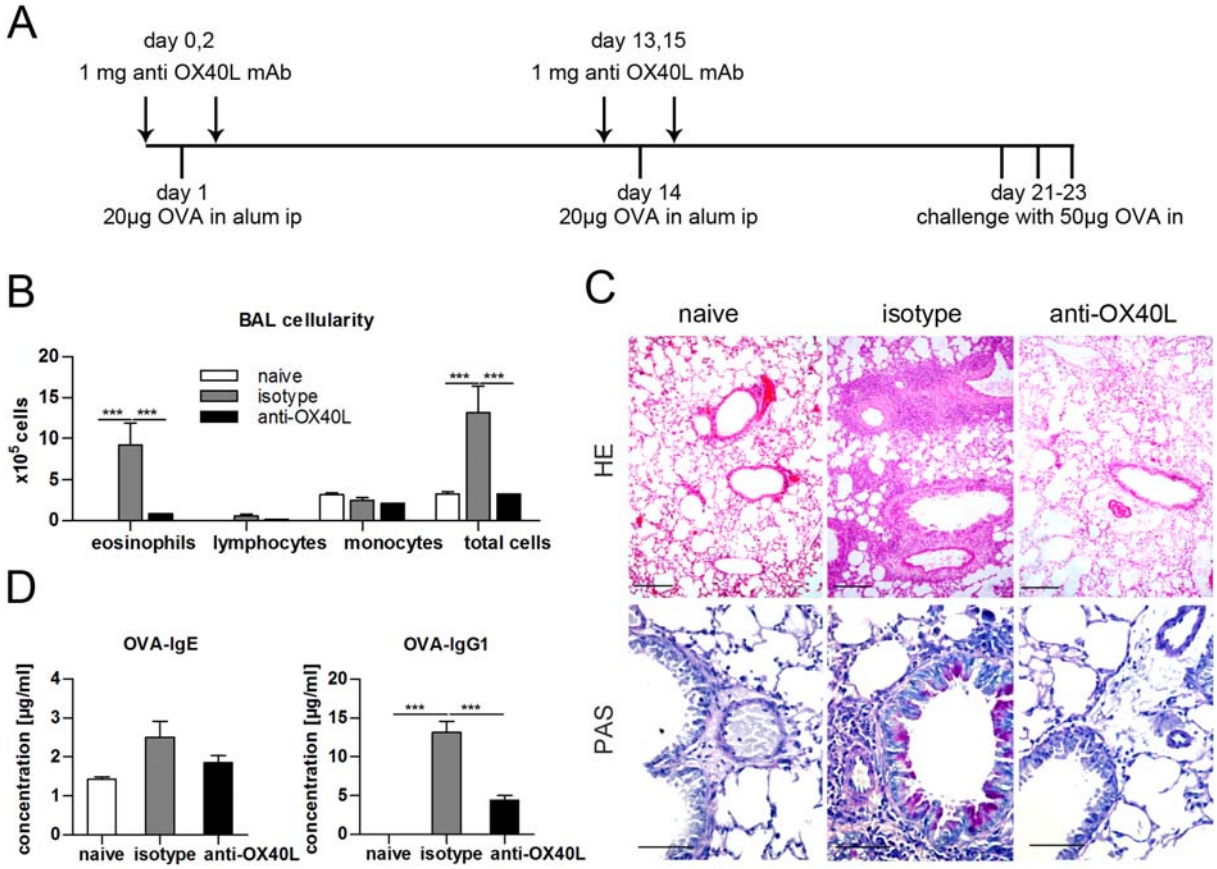

Figure 1. OX40L blockade prevents allergic sensitization. Female Balb/c mice were sensitized with 20 µg OVA-alum given twice in an interval of 14 days followed 7 days later by intranasal challenge for 3 consecutive days with 50 µg OVA per day. Blocking anti-OX40L mAb was applied during sensitization phase. Naive mice were not subjected to any treatment. (A) Immunization and treatment protocol. (B) Differential cell counts were performed on cytospinned BAL samples. (C) Histological sections of lung tissue were stained with hematoxilin/eosin (upper row, bar: 50 µm) and PAS (lower row, bar: 20 µm). (D) Quantification of serum OVA-specific IgG1 and IgE by ELISA. Data are representative of two independent experiments, n=6 per group. *** P<0.001 (two-tailed, students t test).

Treatment with OX40L mAb also prevented the histological changes observed in the lungs, leading to a decrease in inflammatory infiltrates (Figure 1C, upper row) and absence of goblet cell hyperplasia (Figure 1C, lower row). Furthermore, anti-OX40L treatment prevented effective generation of Th2-driven OVA-specific IgG1 and IgE (Figure 1D). We could not detect Th1-driven antigen-specific IgG2a in any animal (data not shown).

Allergic airways inflammation is not only characterized by inflammatory infiltrates in the airways, but also by the production of Th2 cytokines like IL-4, IL-5 and IL-13. We found that the levels of IL-4, IL-5 and IL-13 in the lung tissue of anti-OX40L treated mice were reduced compared to animals not treated with the therapeutic mAb (Figure 2A).

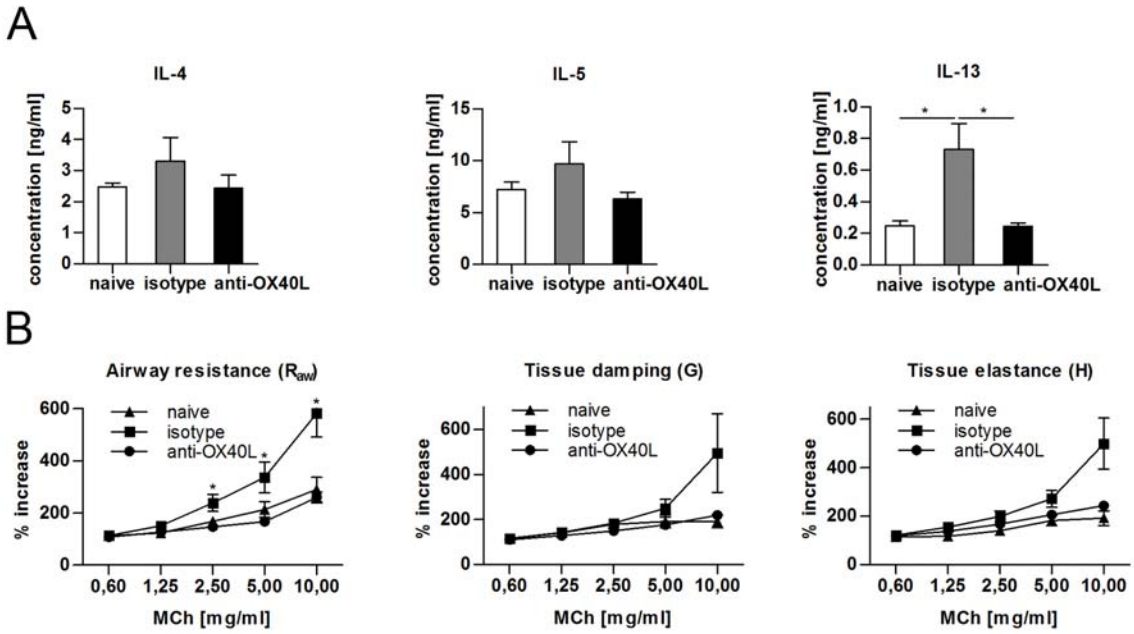

Figure 2. OX40L blockade reduces Th2 cytokines and prevents airway hyperreactivity. Balb/c mice were sensitized as described above. (A) ELISA assays were performed on lung homogenates to quantify the concentration of Th2 cytokines IL-4, IL-5 and IL-13. (B) Invasive measurement of lung function in response to increasing doses of inhaled methacholin (MCh). Data are representative of two independent experiments, n=6 per group. * P<0.05 (two-tailed, students t test).

To assess the impact of anti-OX40L treatment on lung function, we measured airway hyperreactivity in response to increasing doses of the bronchconstrincting agent methacholin (MCh). Lung function was measured using low frequency forced oscillations and partitioned into components representing the airways (resistance), lung parenchyma (tissue damping) and the inverse of tissue elastance. Animals treated with anti-OX40L showed a reduced airway hyperreactivity (Figure 2B).

2.3.2 Anti-OX40L treatment reduces allergic airway inflammation in pre-sensitized mice

The above experiments strongly indicate a potential role for anti-OX40L mAb as a therapeutic agent in the treatment of allergic airway inflammation. Data from our lab showed that treatment with a blocking anti-CD4 mAb is also effective in presensitized mice (Agua-Doce and Graca, 2011). Therefore we investigated whether treatment with anti-OX40L mAb could also prevent allergic airway disease in pre-sensitized animals.

We sensitized Balb/c mice, as described before, to OVA and at day 50 we repeated the sensitization protocol but in presence of OX40L blocking antibody (Figure 3A). Mice exposed to OVA before OX40L treatment only showed a slight reduction in BAL eosinophils compared to untreated mice (Figure 3B).

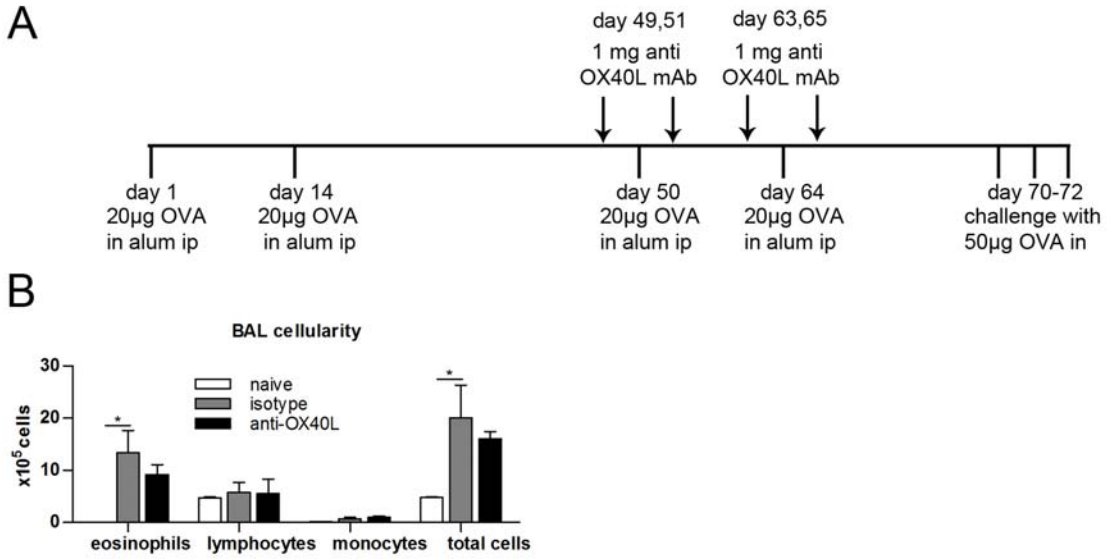

Figure 3. OX40L blockade looses effectivity when administered to pre-sensitized mice. (A) Balb/c mice were sensitized with 20 µg OVA-alum given twice in an interval of 14 days and then resensitized during OX40L blockade from day 50 onwards, which was followed by intranasal challenge for 3 consecutive days with 50 µg OVA per day (B) Differential cell counts were performed on cytospinned BAL samples. Data are representative of two independent experiments, n=6 per group. * P<0.05, ** P<0.01 (two-tailed, students t test).

2.3.3 Anti-OX40L treatment does not lead to long term tolerance

As our initial experiments showed that anti-OX40L treatment can prevent an inflammatory response to OVA, we investigated if anti-OX40L treatment would induce long-term tolerance to OVA, thus preventing subsequent immune responses to the same allergen. For this purpose, we immunized mice in the presence of anti-OX40L mAb and from day 50 onwards, after clearance of the Ab, the animals were re-sensitized and challenged with the same antigen (Figure 4A).

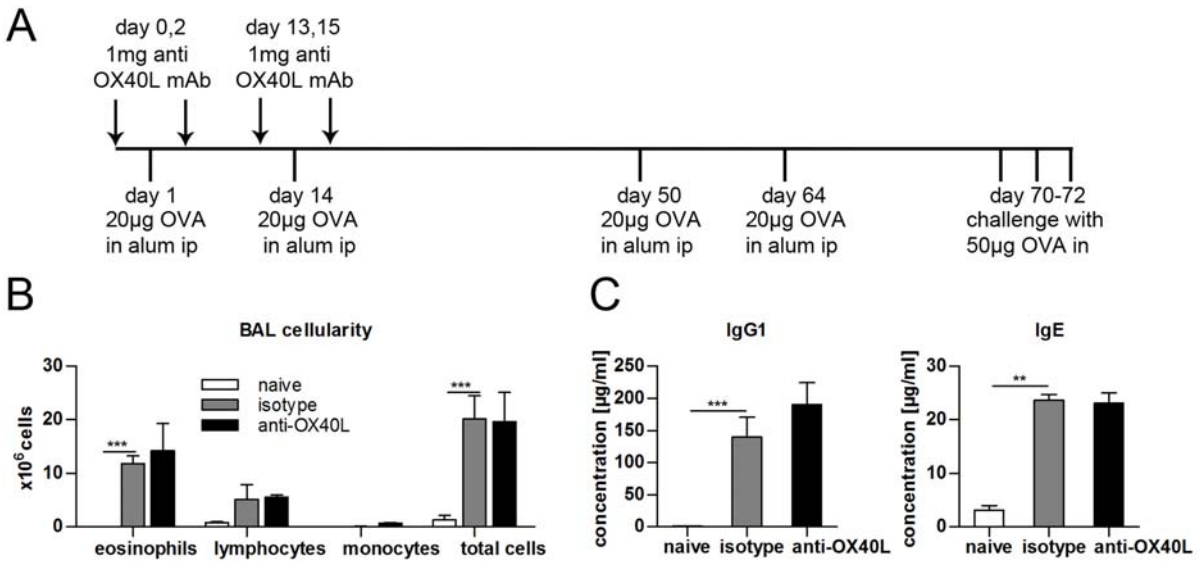

Figure 4. OX40L blockade does not protect mice from subsequent sensitization with the same antigen. (A) Balb/c mice were sensitized with 20 µg OVA-alum given twice in an interval of 14 days during OX40L blockade and then re-sensitzed from day 50 onwards, which was followed by intranasal challenge for 3 consecutive days with 50 µg OVA per day. (B) Differential cell counts were performed on cytospinned BAL samples. (C) Quantification of serum IgG1 and IgE by ELISA. Data are representative of two independent experiments, n=6 per group. ** P<0.01, group. *** P<0.001 (two-tailed, students t test).

We also found that, anti-OX40L treated animals were not protected from development of inflammatory infiltrates in the BAL following subsequent exposure to the same antigen. Moreover, when analyzing serum IgE and IgG1 level, we found anti-OX40L treated animals showed no reduction in those immunoglobulins.

2.4 Discussion

Asthma is a very common disease and its incidence is increasing globally, placing an increasing burden on health services in industrialized and developing countries. This disease is characterized by chronic inflammation and massive infiltration of inflammatory cells in the bronchial mucosa along with goblet cell hyperplasia, thickening of the submucosa and mucus production within the bronchioles (Beasley et al., 1989; McFadden and Gilbert, 1992). These cellular aspects of the disease lead to variable and reversible airway obstruction and AHR (Holgate, 1997).

Our data show that treatment with blocking anti-OX40L antibody during the sensitization phase of the allergic response can reduce the manifestations of allergic airways disease, namely airways eosinophilia, and production of IL-4, IL-5 and IL-13 in the lungs, as well as serum anti-OVA IgE and IgG1 levels. Lung histology as well as measurement of the respiratory mechanics in response to inhaled methacholine confirmed that the airways of mice treated with anti-OX40L are morphologically and physiologically similar to healthy unmanipulated controls. Studies using OX40-deficient mice by Jember *et al*. (Jember et al., 2001) support those findings by demonstrating a requirement for OX40 signals in the initial development of allergic asthma, which was subsequently reproduced in OX40L knockout mice (Arestides et al., 2002).

To reveal whether OX40-OX40L interactions may represent clinically relevant targets for preventing recall immune responses in patients with established asthma, we first sensitized animals with OVA and then waited one month before re-sensitizing the animals during OX40L blockade. Although OX40L blockade was able to prevent pathologic manifestation of asthma, when treated at the time of first contact with the allergen, the treatment was not effective in pre-sensitized mice. Compared to mice not treated with anti-OX40L eosinophilic infiltration in the airways was only slightly reduced. These results are inconsistent with a previous report by Salek-Ardakani et al who showed that anti-OX40L treatment was effective in pre-sensitized mice (Salek-Ardakani et al., 2003) . The apparent paradox may be due to differences in the study design and to the different mAb used. Whereas our anti-OX40L mAb is IgG1, Salek-Ardakani et al used an IgG2b mAb. Moreover, in the study by Salek-Ardakani et al, mice were sensitized at day 0 and then rested 24 days before intranasal challenge. In contrast, in our study mice were immunized at days 1 and 14 and then challenged at day 21, 22 and 23. This protocol leads to a stronger effector response. Furthermore, Salek-Ardakani et al administered the anti-OX40L treatment at the time of challenge. In fact Hoshino et al showed in a protocol similar to ours, no effect of OX40L blockade when applied during the time of challenge (Hoshino et al., 2003).

Evidence from asthmatic patients and from animal models of this disease indicate that memory Th2 cells, secreting IL-4, IL-5, IL-9 and IL-13, are a major driving force behind allergic asthma (Wills-Karp, 1999). Our results suggest that OX40-OX40L blockade has only limited value in the treatment of already established disease and it might be due to the inability of OX40L blockade to control memory cells.

Heart graft survival (Curry et al., 2004), as well as survival of xenogenic islets grafts (Honkanen-Scott et al., 2008) and corneal implants (Hattori et al., 2007) is increased following the blockade of OX40-OX40L signaling. Moreover, increase in $OX40^+$ and $OX40L^+$ cells are associated with intestinal inflammation (Malmstrom et al., 2001; Souza et al., 1999) and blockade of OX40-OX40L interactions inhibits colitis in various mouse models (Malmstrom et al., 2001; Takeda et al., 2004). In all these models it is the reduction of T cell activation and increased apoptosis of activated T cells that are responsible for the reduction of the pathology.

When treating mice with OX40-OX40L blockade during initial antigen exposure and re-challenging the mice 30 days later with the same antigen, anti-OX40L treatment did not prevent allergic airways disease during the recall response. Treated mice showed eosinophilic infiltrates in the airways, as well as high IgE and IgG1 serum level, comparable to untreated controls.

Many investigations have established beyond a doubt the involvement of regulatory T cells in the establishment of tolerance (Wing and Sakaguchi, 2010). Interestingly, there are many conflicting results from *in vitro* and *in vivo* studies, with evidence that OX40 signaling is either neutral or can promote or inhibit Treg cell mediated suppression (Golovina et al., 2008; Hippen et al., 2008; Piconese et al., 2008; Takeda et al., 2004; Valzasina et al., 2005). A recent study by the group of Fiona Powrie, examining the role of OX40 influencing the balance between effector T cells and Treg cells, showed that OX40 expression by Treg cells was indispensable for accumulation of Treg cells in the lymphoid organs, as well as the intestines (Griseri et al., 2010).

On the basis of these observations, it is likely that OX40L blockade rescues from the immediate inflammatory reaction, but due to interference with Treg function long term protection cannot be achieved.

It is clear that blocking late costimulatory molecules provides more specific protection than generally suppressing the immune system, but in the case of asthma the suppression achieved by blocking OX40-OX40L signaling seems to be insufficient to establish tolerance and by that a long term effect.

In summary, our data reveal the importance of OX40-OX40L signaling during an acute allergic immune response. However, OX40L blockade seems to prevent the direct inflammatory response to an allergen, but is ineffective in inducing tolerance to a model allergen.

3. Identification of Foxp3$^+$ follicular T cells

3.1 Background

T_{FH} cells were discovered in human tonsils and described as CD4 T cells with a special phenotype that shows high levels of CXCR5 expression (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000). They exhibit effector functions and, as it is common for effector T cells, have low CCR7 expression, but in contrast to other effector cell populations, they are located in SLOs rather than inflamed nonlymphoid parenchyma. More specifically, due to the fact that low CCR7 is concomitant with high level of CXCR5 expression, T_{FH} cells, as they follow the chemokine CXCL13 expressed by follicular stroma, are located within B cell follicles rather than T-cell zones (Campbell et al., 2003). CXCR5 is also expressed by mature B cells and a small proportion of memory T cells, but the highest expression of CXCR5 is found on T_{FH} cells, together with high expression of PD-1 (and ICOS in humans, but not mice), a key molecule for B cell survival and proliferation (Good-Jacobson et al., 2010).

Whereas the original discovery of T_{FH} cells was a important step, the phenotypic characterization given at the time was insufficient to establish T_{FH} cells as a independent T effector lineage next to Treg, Th1, Th2 and Th17 cells (Murphy and Reiner, 2002; Weaver et al., 2007; Zhu and Paul, 2008). The strongest argument against T_{FH} cells as a separate lineage, was the lack of a key transcription factor associated with their development, while differentiation of naïve CD4 T cells into Treg, Th1, Th2 and Th17 cells is tightly regulated by master transcription factors (Zhu et al., 2010). Additionally, it was not certain, if T_{FH} cells possess special characteristics beyond CXCR5 expression. The recent identification of bcl6 as master transcription factor (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009b), together with the proof, that T_{FH} cells are indispensable for B cell help (Johnston et al., 2009; Nurieva et al., 2008; Yu et al., 2009b) and demonstrating the importance of IL-21 for T_{FH} development (Linterman et al., 2010; Nurieva et al., 2008; Zotos et al., 2010) have established T_{FH} cells as a separate lineage.

The stated function of T_{FH} cells is to provide B cell help. More specificially T_{FH} cells are required for the formation and maintenance of GCs and for the generation of most memory B cells and plasma cells. Crosstalk between GC B cells and T_{FH} cells are mediated by interactions through TCR-MHCII/peptide, CD40-CD40L and ICOS-ICOSL (Kelsoe, 1995). When CD40-CD40L interactions are prevented, the on-going GCR stops and GCs dissolve within 24 hours (Kelsoe, 1995). Mice deficient in ICOS or ICOSL also have impaired GC formation and isotype switching (Dong et al., 2001). Triggering of the SHM process in GC B cells is also dependent on CD40-CD40L interactions (Bergthorsdottir et al., 2001). Immunized mice that lack T_{FH} cells show reduced numbers of germinal center B cells, as well as a reduction of antigen-specific antibody (Nurieva et al., 2009).

Antibodies of different isotypes have different effector functions that are important for the control of different classes of pathogens. Class switch factors are required to instruct isotype-specific B cell CSR. Besides direct B-T_{FH} contact, T_{FH} cells also contribute to the GCR

through soluble mediators like IL-4 and IL-21 (Ozaki et al., 2002; Reinhardt et al., 2009). IL-4 is a class switch factor for murine and human IgG1 and IgE and IL-21 is a class switch factor for human IgG3, IgA, and IgG1 (Avery et al., 2008; Pene et al., 2004) and murine IgG1 (Ozaki et al., 2002). Additional cytokines such as IFN-γ and IL-17 are expressed by T_{FH} cells in a context-dependent manner and control switching to suitable isotypes (Johnston et al., 2009; Mitsdoerffer et al., 2010; Reinhardt et al., 2009; Yusuf et al., 2010). The diverse cytokine expression profile of T_{FH} cells makes them appear quite heterogenous. To confuse matters even further, another population of T cells that is located in extrafollicular plasma cell foci is dependent on the transcription factor bcl6 and shows T_{FH} cell-like activity (Poholek et al., 2010). Moreover, Morita et al. identified a subset of circulating $CXCR5^+$ T cells with potent B cell helper activity (Morita et al., 2011). Even though, Bcl6 was identified as master transcription factor of the T_{FH} lineage leading to recognition of T_{FH} cells as a separate T cell lineage, the ontogeny of T_{FH} cells still remains to be fully established. It is unclear whether T_{FH} cells differentiate from T cells shortly after priming, or whether early Th1, Th2 or Th17 cells can adopt follicular differentiation upon exposure to cytokines such as IL-6 and IL-21 in mice and IL-12 in humans. Moreover, due to their differential cytokine expression profile it is to date also not clear, whether T_{FH} cells contain further subpopulations or just consist of one homogeneous population, that depending on the circumstances expresses different cytokines. In this chapter we demonstrate that T_{FH} cells contain a $Foxp3^+$ regulatory subset, which differentiates predominantly from natural Treg cells, and possesses immune suppressive function.

3.2 Materials and Methods

Mice and immunization

Balb/c, C57Bl/6, CXCR5$^{-/-}$, as well as DO11.10 and OT2.Rag2$^{-/-}$ mice, that carry the MHC class II restricted rearranged T cell receptor transgene for OVA peptide antigen, were originally purchased at Jackson Laboratory. Foxp3^{gfp} knockin mice were generously provided by A.Y. Rudensky and TCR$\alpha^{-/-}$ mice by S.Tonegawa.

Unless otherwise stated in the text, animals were immunized intraperitonally with 20 µg OVA emulsified in alum. All mice were kept under specific pathogen-free (SPF) conditions at the Instituto Gulbenkian de Ciencia (IGC) animal facility and used at an age between 6-8 weeks. All animal experiments were conducted according to the institutional animal ethics committee.

Adoptive cell transfer

For adoptive cell transfers single cell suspensions from a pool of spleen and LN were sorted based on expression of CD4, CXCR5, PD-1, CD25, and GFP (cells from Foxp3^{gfp} reporter mice) in a FACS Aria (BD, Franklin Lakes, USA), with doublet exclusion in all experiments. Unless otherwise stated in the text 1x10^4 cells of the indicated cell population were injected intravenously into TCR$\alpha^{-/-}$ mice. In all transfer experiments mice were immunized with OVA-alum one day after adoptive transfer.

Flow Cytometry

To obtain single cell suspension, spleen and LN's were minced through a 45 µm nylon mesh and washed with phosphate-buffered salin (PBS) supplemented with 2% fetal calf serum (FCS). For CXCR5 staining cells were kept at 37°C for 30 min prior to antibody incubation. The Foxp3 staining was performed using the APC Anti-Mouse/Rat Foxp3 staining Set (ebioscience) following the manufacturer's instructions. The following mAb were purchased from ebioscience: PD-1-PE (J43), CD4-APC-Cy7 (L3T4), Thy1.2-PECy7 (53-2.1), Foxp3-APC (FJK-16s), Bcl6-PE (GI191E), GITR-biotin (DTA-1), CD103-biotin (2E7), CD69-biotin (H1.2F3), CD25-PECy7 (PC61.5). CXCR5-biotin (2G8), as well as IgG2a-biotin (R35-95) were purchased from BD Bioscience and CD19-PE from our in-house production.

Suppression assay

Sorted populations of CD4$^+$Foxp3^{gfp+} T cells were co-cultured in Terasaki plates (Greiner, Frickenhausen, Germany) with gamma-irradiated APCs, and CD4$^+$Foxp3^{gfp-} effector T cells (1:3:1 ratio). Cultures were supplemented with 2.5 µg/ml soluble anti-CD3 for 3 days, with addition of 1 µCi ^{3}H-thymidine (Amersham, Sunnyvale, CA) in the last 12h.

Foxp3 conversion assay

T_{FH} cells ($CD4^+CXCR5^+PD1^+Foxp3^-$) and non-follicular CD4 effectors ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^-$) were sorted from spleen and LN's of $Foxp3^{gfp}$ reporter mice. $5x10^4$ cells were cultured in triplicates in 96 flat-bottom plates (TPP, Switzerland) with plate bound 3 µg/ml anti-CD3 Ab (145-2C11, ebiosciences). Cultures were supplemented with 2 µg/ml soluble anti-CD28 Ab (ebiosciences) and 5 ng/ml soluble TGFβ (R&D Systems) or without TGFβ Ab for 3 days.

Confocal Microscopy

20 µm cryosections fixed in acetone (Sigma) and 50 µm vibratome sections from PFA (Sigma) fixed tissue were obtained from mesenteric lymph nodes (mLN). For Foxp3 staining sections were permeabilized with the Fix/Perm buffer from the Foxp3 staining Set (eBioscience). Samples were stained with the following primary antibodies: rabbit anti-CD3, anti-GFP; rat anti-CD3 (Abcam), anti-Foxp3 (ebioscience), anti-CD4-alexa647 (Serotech); goat anti-IgM-TxRd (SouthernBiotech, Birmingham, USA); and mouse anti-DO11.10 clonotypic TCR (Caltag, Carlsbad, USA); as well as PNA-FITC and PNA-bio (Vector, Burlingam, USA). As secondary antibodies we used: anti-FITC-alexa488, anti-rabbit-alexa488, anti-rabbit-alexa647, anti-rat-alexa633 (Invitrogen, Carlsbad, USA); and avidin-rhodamin (Vector) and streptavidin-DyLight488 from ThermoScientific (Massachusetts, USA). Images were acquired using a LSM710 confocal microscope (Zeiss, Jena, Germany) equipped with a 5x (0,16 NA, Zeiss), 10x (0,30 NA, Zeiss), 20x (0,80 NA, Zeiss) and a 40x (1,30 NA, Zeiss) objective. Image analysis was performed using LSM Image Browser.

Statistical analysis.

Statistical significance was determined using the two-tailed non-parametric Mann-Whitney test and P values <0.05 were deemed significant (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

3.3 Results

3.3.1 Follicular CD4$^+$ T cells contain a Foxp3$^+$ subset

Follicular CD4$^+$ T cells have been shown to produce different types of cytokines depending on the context they are studied in, representing a “Th1-like”, “Th2-like” or “Th17-like” phenotype (Johnston et al., 2009; Mitsdoerffer et al., 2010; Reinhardt et al., 2009; Yusuf et al., 2010). Moreover, it has been shown that regulatory T cells are able to suppress B cell proliferation as effectively as they suppress T cells (Lim et al., 2005). We therefore investigated whether follicular CD4$^+$ T cells also contain cells with a “Treg-like” phenotype.

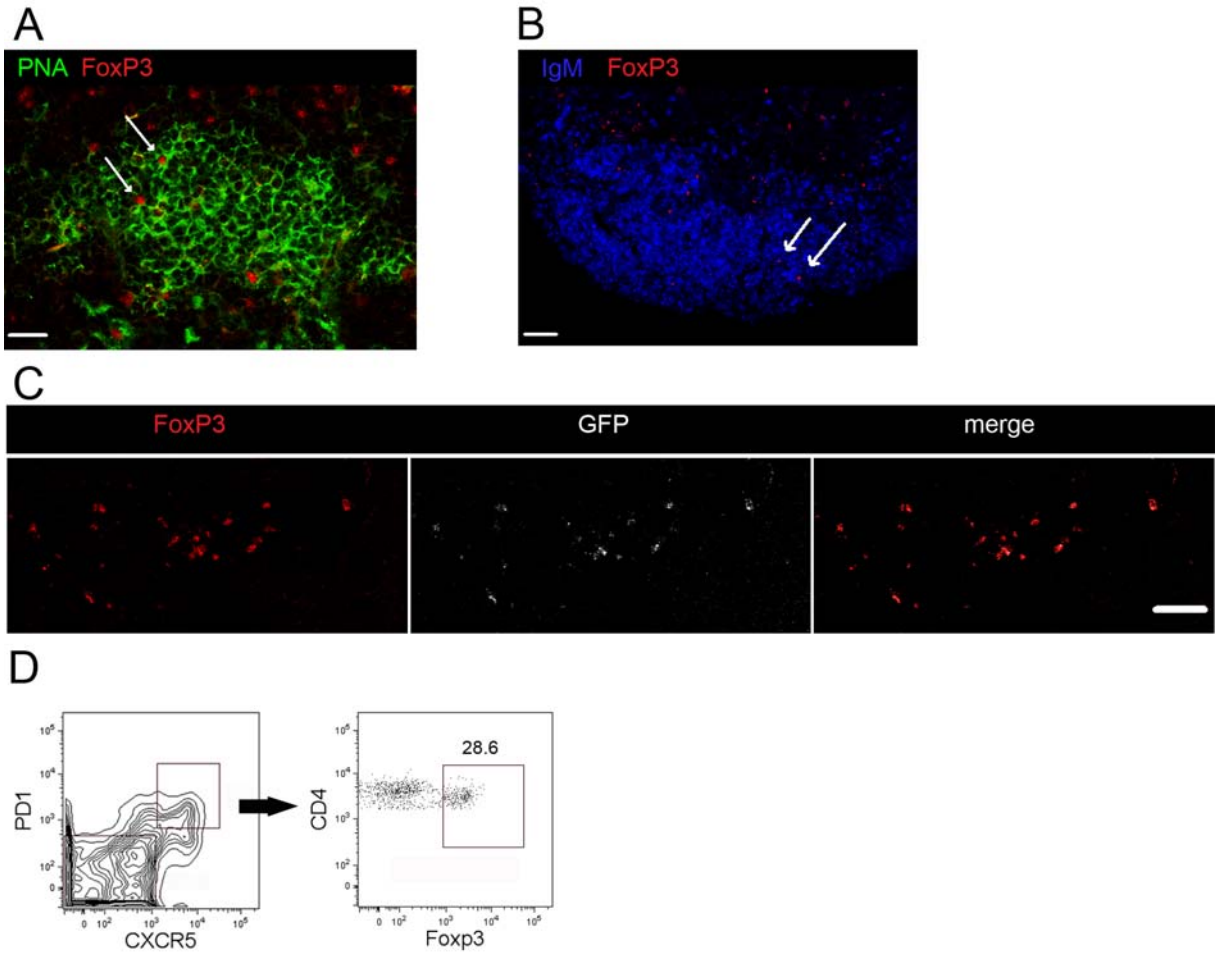

Figure 1. Foxp3$^+$ T cells can be found within B cell follicles and GC. Cryosections of mLNs were stained with (A) PNA and anti-Foxp3 to identify Foxp3$^+$ cells within the GC (scale bar represents 20 µm) or (B) IgM and anti-Foxp3 to identify Foxp3$^+$ cells within the B cell follicle (scale bar represents 50 µm). (C) Cryosections from mLNs of Foxp3^{gfp} knock-in mice were stained with anti-Foxp3 and anti-GFP antibody to confirm that both molecules allowed the identification of the same cells (scale bar represents 50 µm). (D) Lymphocytes from mLN of mice immunized with OVA-alum 12 days before were studied by flow cytometry. T_{FH} cells were defined as being CD4$^+$CXCR5$^+$PD1$^+$. The right plot shows CD4 and Foxp3 expression within the T_{FH} gate. Data are representative of two independent experiments.

Using confocal microscopy we could detect Foxp3$^+$ T cells in B cell follicles, as well as in GCs of mLNs (Figure 1A,B). We confirmed the reliability of Foxp3 staining using Foxp3^{gfp} knock-in mice (where Foxp3 and GFP are fused together), showing a perfect overlap of Foxp3 and GFP staining (Figure 1C).

Furthermore we could confirm the existence of a Foxp3$^+$ subpopulation within follicular CD4$^+$ T cells in mLNs (Figure 1D), as well as the spleen (data not shown), by flow cytometry.

3.3.2 Follicular Foxp3$^+$ T cells share properties of Foxp3$^+$ Treg cells and T_{FH} cells

To further characterize the follicular Foxp3$^+$ T cells, we analyzed follicular CD4$^+$ T cells by flow cytometry from mLNs, 12 days after immunization with OVA. Follicular Foxp3$^+$ T cells were identified as CD4$^+$CXCR5$^+$PD-1$^+$Foxp3$^+$ cells, while the non-follicular counterparts were identified as CD4$^+$CXCR5$^-$PD-1$^-$Foxp3$^+$ cells (Figure 2A,B). We found that follicular Foxp3$^+$ T cells share the expression of several markers with "conventional" non-follicular Treg cells, such as high levels of CD25, GITR, or CD103. However follicular Foxp3$^+$ cells also represent molecules characteristic of T_{FH} cells, such as CXCR5 and PD-1, as well as the lineage-specific transcription factor Bcl-6 (Figure 2B). Foxp3$^+$ follicular T cells also expressed high levels of CD69, suggesting an activated phenotype.

Importantly, Bcl-6 was recently identified as the master transcription factor for the T_{FH} lineage, by turning on a wide-spread gene repressor program acting on key transcriptional regulators of other T helper cell lineages, namely Tbet (Th1) and RORγt (Th17), as well as a large number of miRNAs (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009b). However, a repressive function towards the Treg lineage, or its key regulator Foxp3, was never described.

Therefore, Foxp3$^+$ T cells found in GCs and B cell follicles appear to represent a regulatory subpopulation of follicular T cells, bearing a distinctive phenotype from the non-follicular conventional Treg cells. In support of that conclusion, sorted follicular Foxp3$^+$ T cells (based on CD4, CXCR5, PD-1 and Foxp3) had the same suppressive activity, as their non-follicular counterparts (Figure 2C).

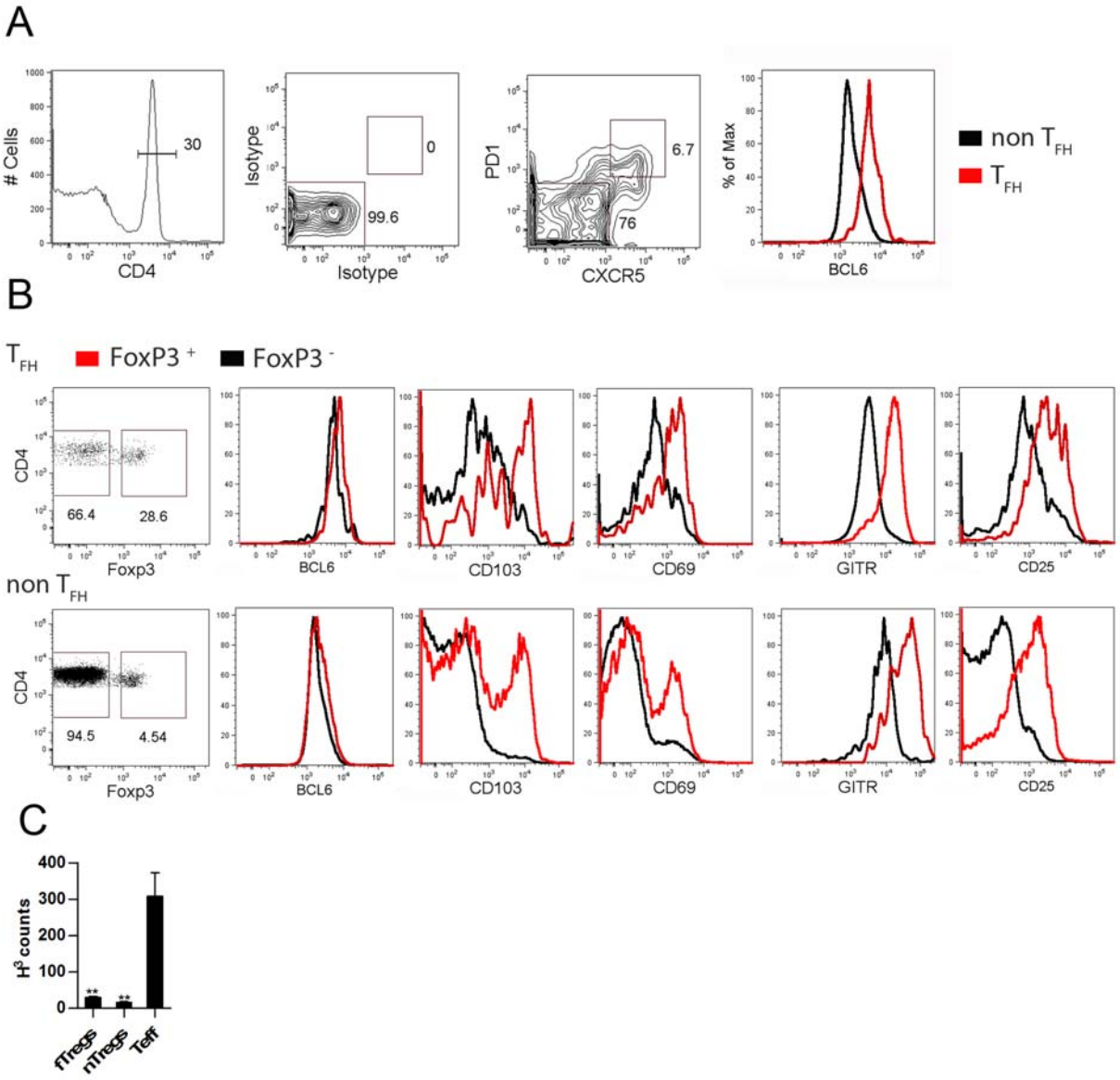

Figure 2. Follicular Foxp3$^+$ T cells share propperties of T_{FH} and Treg cells. Lymphocytes from mLNs of Balb/c mice were studied by flow cytometry 12 days post-immunization with 20 µg OVA. (A) T_{FH} cells were defined as being CD4$^+$CXCR5$^+$PD-1$^+$ as shown by the represented gate. Non-follicular T cells (non-T_{FH}) were defined as CD4$^+$CXCR5$^-$PD-1$^-$ cells. (B) The expression of the indicated molecules was compared between Foxp3$^+$ and Foxp3$^-$ T cells within the T_{FH} (upper row) and non-T_{FH} gate (lower row). (C) Follicular Foxp3$^+$ T cells (CD4$^+$CXCR5$^+$PD1$^+$Foxp3$^+$) and non-follicular conventional Treg cells (CD4$^+$CXCR5$^-$PD-1$^-$Foxp3$^+$) were sorted from spleen and LN's of Foxp3^{gfp} reporter mice. 2x10^3 Treg cells were co-cultured in triplicate with gamma-irradiated APCs and Foxp3^{gfp-} effector cells (1:3:1 ratio) in Terazaki plates. Cultures were supplemented with 2.5 µg/ml soluble anti-CD3 Ab for 3 days and ^{3}H-thymidine was added in the last 12 hours. Data are representative of two independent experiments. * P<0.05, ** P<0.01 (Mann-Whitney non-parametric, two-tailed test).

3.3.3 Specificity of follicular Foxp3$^+$ T cells

At day 4 post-immunization with OVA-alum, antigen-specific T cells could be found in great quantities in mLNs (Figure 3A). To investigate the presence of antigen-specific follicular Foxp3$^+$ T cells, we adoptively transferred 5x10^7 OVA-specific cells from DO11.10 mice into

Balb/c mice, immunized with 20 µg OVA on the following day. Transferred antigen-specific effector T cells could be readily detected in mLN and GCs at day 4 of GCR (Figure 3A, B). Moreover, we were also able to detect Foxp3$^+$ antigen-specific T cells within the GCs (Figure 3B). Future experiments will have to investigate whether Foxp3$^+$ follicular Tcells show specificity towards the antigen that triggered the GCR.

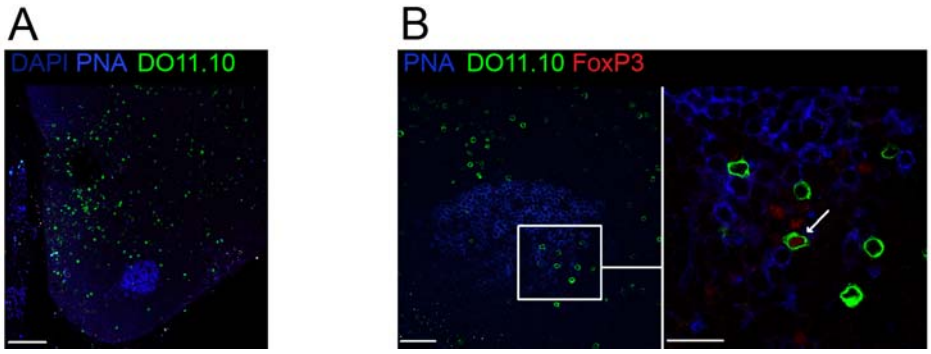

Figure 3. Antigen-specific Foxp3$^+$ T cells can be detected in GCs. 5x10^7 OVA specific cells from DO11.10 mice were transferred into Balb/c mice and subsequently immunized with 20 µg OVA. Cryosections of mLNs were stained with (A) DAPI, PNA and anti-DO11.10 clonotypic TCR Ab to identify OVA-specific cells (scale bar represents 100 µm) or (B) with PNA, anti-KJ and anti-Foxp3 to identify OVA-specific Foxp3$^+$ cells within the GC (scale bar of the left image represents 50 µm, right image 20 µm). Data are representative of two independent experiments.

3.3.4 Origin of follicular Foxp3$^+$ T cells

The follicular Foxp3$^+$ T cell population showed combined markers of conventional non-follicular Treg cells and T_{FH} cells. Therefore, we investigated, how follicular Foxp3$^+$ T cells develop and which cells represent their progenitors.

We confirmed, as anticipated, that the thymus does not contain a population of Foxp3$^+$ T cells with follicular characteristics (Figure 4A), indicating that follicular Foxp3$^+$ T cells acquire their phenotype in the periphery, as it is the case with T_{FH} cells (Crotty, 2011).

Foxp3$^+$ regulatory T cells can be distinguished as natural Tregs, when they are derived from the thymus or induced Tregs, when they originate from conversion of CD4 cells in the periphery (Chen et al., 2003). Addition of TGFβ to *in vitro* cultures is commonly used to convert CD4$^+$Foxp3$^-$ cells into CD4$^+$FoxP3$^+$ iTregs (Oliveira et al., 2011a).

We sorted T_{FH} and conventional CD4 T cells from mLNs and spleen and stimulated them *in vitro* with plate-bound anti-CD3 in presence of anti-CD28 and TGFβ, we found that T_{FH} cells are resistant to conversion towards a Foxp3$^+$ phenotype (Figure 4E). As expected around 50% of the CD4 T cells started expressing Foxp3 together with CD25 after 3 days of culture (Figure 4E).

To further confirm those observations and in order to establish the origin of follicular Foxp3$^+$ cells, we adoptively transferred sorted T_{FH} or conventional CD25$^-$ CD4$^+$ T cells from wild-type

mice, together with PD-1$^-$CXCR5$^-$Foxp3^{gfp+} natural Treg cells into TCRα$^{-/-}$ mice that were subsequently immunized with OVA-alum. Since, adoptive transfer of a CD4 population

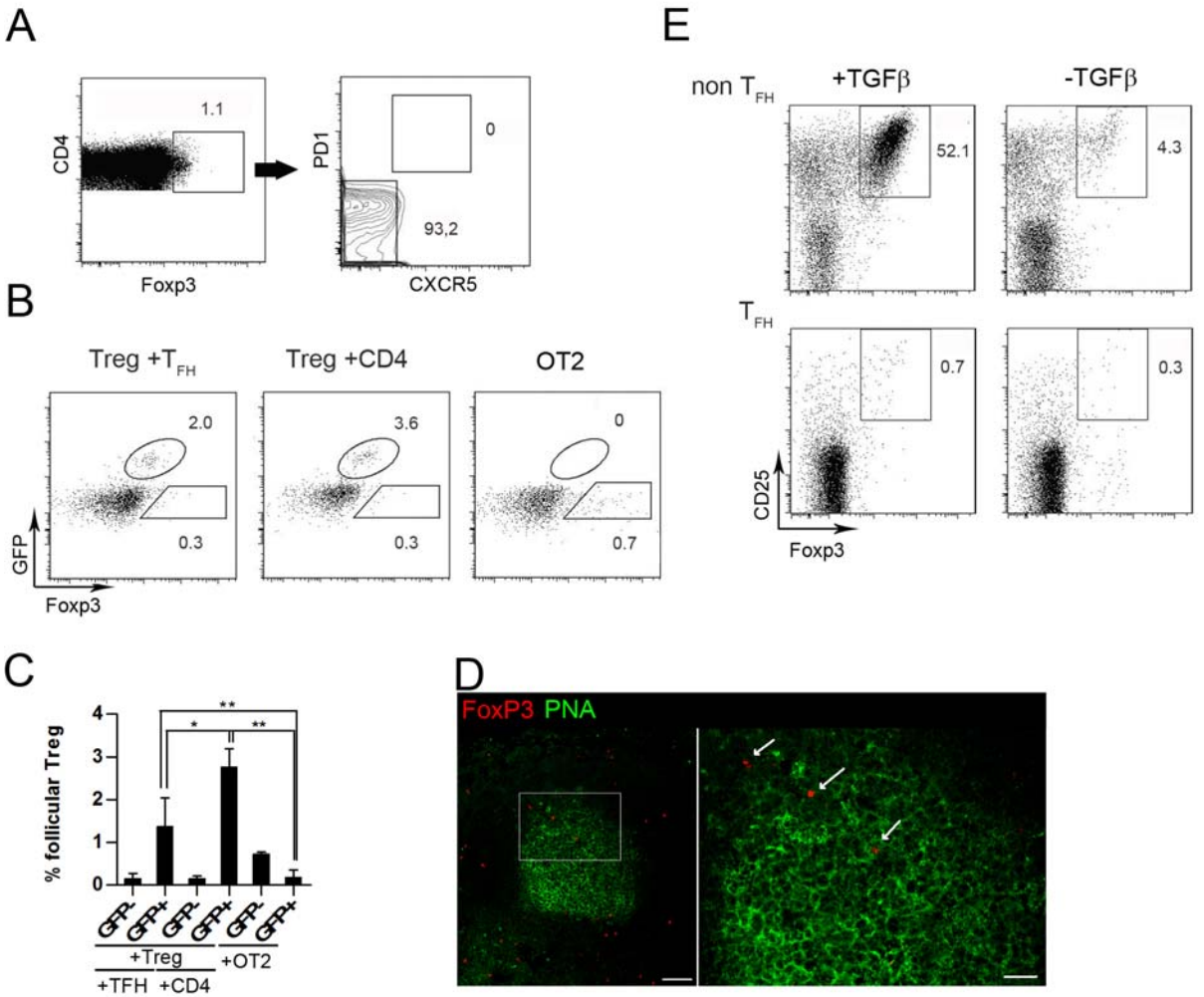

Figure 4. Follicular Foxp3$^+$ T cells originate from non-follicular FoxP3$^+$ T cells. (A) CD4$^+$Foxp3$^+$ thymocytes from C57Bl/6 mice were studied by flow cytometry for CXCR5 and PD1 expression. . (B) TCRα$^{-/-}$ mice were adoptively transferred with equal numbers of PD-1$^-$ CXCR5$^-$ Foxp3$^+$ Treg cells sorted from Foxp3^{gfp} knock-in mice, and CD4$^+$CD25$^-$ T cells (T_{FH}: PD-1$^+$CXCR5$^+$ or CD4: PD-1$^-$CXCR5$^-$) from B6 mice. Control mice were transferred with Foxp3$^-$ OT2 cells alone. All mice were immunized with OVA-alum. The dot plots represent follicular CD4$^+$ T cells, gated in the PD-1$^+$CXCR5$^+$ region, analyzed for Foxp3 and GFP expression. (C) Frequency of GFP$^-$ and GFP$^+$ follicular Foxp3$^+$ cells. (D) Micrograph depicting Foxp3$^+$ cells within the GC, left bar: 50 µm, right bar: 20 µm. (E) Foxp3^{gfp-} T_{FH} (sorted as CD4$^+$PD-1$^+$CXCR5$^+$) and non-T_{FH} cells (sorted as CD4$^+$PD-1$^-$CXCR5$^-$) were cultured for 3 days with plate-bound anti-CD3 in presence or absence of 5 ng/mL TGFβ. The frequency of cells converted into Foxp3$^+$ cells was analyzed at the end of the culture. Data are representative of two independent experiments, n=3 per group * P<0.05, ** P<0.01 (Mann-Whitney non-parametric, two-tailed test).

devoid of Treg cells does lead to inflammatory bowel disease (Read et al., 2000), control mice that did not receive Treg cells were transferred with OT2 cells, as these TCR-transgenic T cells do not lead to autoimmunity in the absence of Tregs. We found in both cases that the majority of follicular Foxp3$^+$ T cells recovered from the TCRα$^{-/-}$ mice had derived from the transferred Treg population, as GFP was expressed together with Foxp3 (Figure 4B,C). Intriguingly, that fraction was larger when CD4 cells were transferred together with Foxp3^{gfp+}

Treg cells. Furthermore, the presence of Foxp3$^+$ cells within the GC could be confirmed by confocal microscopy (Figure 4D).

Analysis of the T_{FH} cells in both transfer systems also revealed percentages of T_{FH} cells in mLN in CD4 transferred mice almost as high as in T_{FH} transferred mice, suggesting that non-follicular CD4 effector cells can easily convert to T_{FH} cells, during the course of a secondary immune response to OVA (data not shown).

Taken together, these data suggest that follicular Treg cells derive predominantly from natural Treg cells, while T_{FH} cells are not amenable to TGFβ-dependent conversion into Foxp3$^+$ regulatory cells.

3.4 Discussion

T_{FH} cells provide help to B cells and allow formation of long-lived antibody responses. Despite an improved understanding of the molecular program that drives T_{FH} cell formation, CD4 T cells in B cell follicles are heterogeneous (Vinuesa et al., 2005; Yu et al., 2009a), and the point in ontogeny where a CD4 cell becomes a T_{FH} cell is still not clearly defined. In this study we have identified a suppressive $Foxp3^+$ subpopulation within follicular $CD4^+$ T cells, which we denote as $T_F$reg cells, thus broadening the heterogeneity of follicular T cells.

$FoxP3^+$ Treg cells are important to maintain immune tolerance and prevent inflammatory diseases (Sakaguchi et al., 2008). Recently, it has become clear that Treg cells can be divided into several distinct subsets, with unique functional and homeostatic properties, that work in concert to maintain normal immune homeostasis (Feuerer et al., 2009). Follicular $Foxp3^+$ T cells could represent an additional regulatory subset, since they share several markers with conventional $Foxp3^+$ Tregs. Besides expressing the transcription factor for the Treg lineage Foxp3, they are also positive for CD25, GITR and CD103 (αEβ7 integrin). CD103, as well as CD25 and GITR are characteristically expressed by natural Treg cells (Lehmann et al., 2002; Sakaguchi et al., 1995; Shimizu et al., 2002). Furthermore, CD103 was shown to be important for the homing of Treg cells to the gut and skin (Weiner et al., 2011). Our study was performed in mLNs. Therefore CD103 might be, in addition to CXCR5, an important homing receptor for follicular regulatory T cells. Many other chemokine receptors have been implicated in the inflammatory recruitment of Treg cells in different immunological settings, (Campbell and Koch, 2011). Indeed, Treg cells probably use a combination of homing molecules that can function redundantly to control their migration during inflammatory responses. Expression of the homing receptor CXCR5 is also commonly used to identify T_{FH} cells. However, it is the combination with PD-1 (and ICOS in humans, but not mice), and more importantly, the amount of CXCR5 and PD-1 expressed, that is useful to identify T_{FH} cells. High expression of CXCR5, together with PD-1 allowed the identification of follicular $Foxp3^+$ T cells that also expressed high levels of Bcl6, the key transcription factor for the T_{FH} cell lineage (Yu et al., 2009b). Thus, at this point it is difficult to decide whether follicular $Foxp3^+$ T cells represent a regulatory subpopulation of the T_{FH} lineage or a follicular subpopulation of the Treg lineage, since they share characteristics of both lineages, including the master transcription factors. Interestingly, Bcl-6 was identified in 2009 as the master transcription factor for the T_{FH} lineage, by inducing a wide-spread gene repressor program inhibiting key transcriptional regulators of other T helper cell lineages, namely Tbet (Th1) and RORγt (Th17), as well as a large number of miRNAs. However, a repressive function towards the Treg lineage, or its key regulator Foxp3, was not found (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009b).

Lim et al described that human tonsils have a $CD69^-CD4^+CD25^+$ regulatory T cell population able to suppress antibody production of $CD19^+$ B cells, but not able to migrate to the B cell follicle. Interestingly they also studied, in the same report, a $CD25^+CD69^+$ population with no regulatory function, but the ability to migrate to the B cell follicle (Lim et al., 2004). Our data show follicular $Foxp3^+$ Treg cells express high amounts of CD69 and are as suppressive as

conventional non-follicular Treg cells. Moreover the presence of follicular Treg cells in the B cell follicle could be confirmed by confocal microscopy.

Non-follicular Foxp3^{+} Treg cells can be distinguished in natural Treg cells, which originate in the thymus and induced Treg cells that originate from peripheral CD4 T cells. TGFβ is crucial to peripheral conversion of CD4 T cells into iTreg cells and activation of CD4 T cells in the presence of TGFβ is frequently used to induce regulatory FoxP3^{+} T cells (Chen et al., 2003; Oliveira et al., 2011a). Much to our surprise, when adding TGFβ to in vitro cultures of T_{FH} cells we could not induce Foxp3 expression. Moreover, when we transferred OT2 cells into TCRα$^{-/-}$ mice we could not find *in vivo* conversion of those Foxp3^{-} cells into follicular Treg cells. However, when we transferred natural Treg cells together with T_{FH} or CD4 T cells, some of the natural Treg cells acquired access to the follicles and phenotypic follicular characteristics.

Previous data from Tsuji et al showed that natural Treg cells can originate T_{FH} cells in the Peyer's Patches (Tsuji et al., 2009). Our data indicate that in the same way, as Treg cells can originate T_{FH} cells some Treg cells can also differentiate towards a follicular phenotype and keep their regulatory profile, however our experiments do not exclude the possibility of T_{FH} cells acquiring a regulatory phenotype.

GC regulation or malfunctions are linked to the onset of severe diseases, as cancer and autoimmunity (Vinuesa and Cook, 2001). Taken together, our data show that follicular CD4^{+} T cells include a new regulatory subpopulation, which may ultimately impact the prevention of such pathologies by controlling the GCR.

4. Regulation of GCR by Foxp3$^+$ follicular T cells

4.1 Background

GCs are temporary structures generated within B cell follicles in SLO during immune responses to foreign antigens. Antigen-specific GC B cells undergo extensive proliferation, CSR and SHM, an unique process whereby they have the potential to acquire higher affinity for the antigen (Berek et al., 1991). Along several cell divisions, high affinity GC B cells are selected and differentiate into either memory cells or long-lived plasma cells, providing the basis of a process called affinity maturation of serum antibodies (Eisen and Siskind, 1964). Long-term humoral immunity, as it is achieved in many prophylactic vaccines, is provided by those plasma cells and memory B cells and is a critical component to protect the body during subsequent infection (Radbruch et al., 2006).

Mature B cells continuously recirculate through SLO in search of signs of infection and, on reaching the follicles, move rapidly within them to survey these areas for antigen. On antigen encounter, B cells initially gather at the boundary between B-cell follicles and T-cell areas (Garside et al., 1998). This movement is directed by the rapid upregulation of the chemokine receptor CCR7 consequent on antigen activation and the expression of the CCR7 ligand CCL19 in the T cell area (Reif et al., 2002) and facilitates cognate encounters with T cells at the T-B boundary. Those encounters drive initial B-cell proliferation and are required for the induction of GCs. Notably, the interaction of the TNF receptor family member CD40, which is constitutively expressed by B cells, and its ligand, CD40L (CD154), which is expressed by activated CD4 T cells, is crucial for formation of GCs (Foy et al., 1994b; Han et al., 1995). Thus GCs are believed to be heavily dependent on CD4 T cells. In addition to cellular interaction with B cells, activated CD4 T cells also secrete soluble mediators like cytokines to drive B-cell proliferation as well as differentiation. Cytokine signals play a central role in triggering the molecular events that lead to immunoglobulin CSR and thus the production of functionally higher specialized antibody isotypes like IgG, IgE and IgA. After initial activation, proliferating B-cell blasts proceed to one of two independent pathways of migration and differentiation (Jacob et al., 1991a; Liu et al., 1991b). Responding B cells can either migrate from the T-B boundary to extrafollicular areas, where they are induced to rapidly expand and differentiate into plasmablasts and plasma cells. These transient antibody-secreting cells provide the most immediate source of antigen-specific antibodies and provide rapid protection before the slower GC response is established (MacLennan et al., 2003). Alternatively, antigen-engaged B cells migrate back to adjacent B-cell follicles, where they seed GCs (Allen et al., 2007a).

Deregulation of proliferation, mutation and differentiation in GCs can lead to detrimental outcomes, including oncogenesis and immunodeficiency (Crotty, 2011; Linterman et al., 2009). Moreover, it has been shown that mutant B cells with self-specificity can be supported in GCs and contribute to autoimmunity (Sims et al., 2001). Therefore, regulation

of the quality and quantity of plasma cells and memory B-cell populations in GCs is very important to prevent immunopathology.

How this regulation is achieved remains poorly understood. A potential mechanism for this could be the presence of some regulatory T cells within the follicular T cell population in GCs. Moreover, theoretical modeling points to processes related to T-cell kinetics as being the dominant steps in the GC dynamics (Moreira and Faro, 2006). This led us to propose the hypothesis, that regulatory mechanisms affecting antigen-specific T_{FH} cells and/or antigen-specific GC B cells do exist. A possible basis for some of the required GC regulation would be the involvement of $Foxp3^+$ regulatory T (Treg) cells during a GCR.

Currently, little is known about Treg cells in connection with GCs. Some Treg cells are known to express high levels of CXCR5 and to display positive chemotaxis toward a CXCL13 gradient *in vitro* (Lim et al., 2004). Furthermore, $CD25^+$ T cells were already shown to be present in the GCs of human tonsils and to suppress *in vitro* T cell activation and immunoglobulin production by B cells (Lim et al., 2005; Lim et al., 2004). Moreover, we demonstrated in chapter 3, that follicular $CD4^+$ T cells include a $Foxp3^+$ regulatory subpopulation.

Here we show that after immunization with a TD antigen follicular $Foxp3^+$ Treg cells in murine mLNs participate in the GC regulation, contributing to both the dynamics and the amplitude of the GCR. Furthermore follicular Treg cells had an impact on antibody production and CSR, suggesting that they play an important role in the regulation of GCRs.

4.2 Materials and Methods

Mice and immunization

Balb/c, C57Bl/6, CXCR5$^{-/-}$, as well as OT2.Rag2$^{-/-}$ mice, that carry the MHC class II restricted rearranged T cell receptor transgene specific for OVA peptide antigen, were originally purchased at Jackson laboratory. Foxp3^{gfp} knockin mice were generously provided by A.Y. Rudensky and TCR$\alpha^{-/-}$ mice by S.Tonegawa.

Unless otherwise stated in the text, animals were immunized i.p. with 20 µg of OVA (Sigma, St Louis, USA) previously run through a DetoxyGel column (Pierce, Rockford, USA) in 2.0 mg of endotoxin-free aluminum hydroxide (alum, Alu-gel-S, Serva, Heidelberg, Germany).
All mice were kept under specific pathogen-free (SPF) conditions at the Instituto Gulbenkian de Ciencia (IGC) animal facility and used at an age between 6-8 weeks. All animal experiments were conducted according to the institutional animal ethics committee.

Adoptive transfer

For adoptive cell transfers single cell suspensions from a pool of spleen and mesenteric lymph nodes (mLN) were sorted based on expression of CD4, CXCR5, PD-1, CD25, and GFP (cells from Foxp3^{gfp} reporter mice) in a FACS Aria (BD, Franklin Lakes, USA), with doublet exclusion in all experiments. Unless otherwise stated in the text 1x10^4 cells of the indicated cell population were injected intravenously into TCR$\alpha^{-/-}$ mice. In all transfer experiments mice were immunized with OVA-alum one day after adoptive transfer.

Flow Cytometry

Single cell suspensions of mLNs were analyzed by flow cytometry using mAb targeting: PD-1 (J43), CD4 (L3T4), Thy1.2 (53-2.1), Foxp3 (FJK-16s), Ly-77 (GL7), CD25 (all from eBiosciences); and CXCR5 (2G8), FAS (Jo2) and Rat IgG2a (R35-95) (BD Bioscience, San Diego, USA). Foxp3 staining was performed using the Foxp3 staining Set (ebioscience) following the manufacturer's instructions.

Confocal Microscopy

20 µm cryosections fixed in acetone (Sigma) and 50 µm or 300 µm vibratome sections from paraformaldehyde (PFA, Sigma) fixed tissue were obtained from mLN. For Foxp3 staining sections were permeabilized with the Fix/Perm buffer from the Foxp3 staining Set (eBioscience). Non-specific binding was blocked with 1% BSA (Sigma) and 3% serum (abcam). Samples were stained with primary antibodies as follows: rabbit anti-CD3, anti-Ki67, anti-GFP; rat anti-CD3 (Abcam), anti-Foxp3 (ebioscience), anti-CD4-alexa647 (Serotech); goat anti-IgM-TxRd (SouthernBiotech, Birmingham, USA); and mouse anti-DO11.10 TCR (Caltag, Carlsbad, USA); as well as PNA-FITC and PNA-bio (Vector, Burlingam, USA). The following

secondary antibodies were used: anti-FITC-alexa488, anti-rabbit-alexa488, anti-rabbit-alexa647, anti-rat-alexa633 (Invitrogen, Carlsbad, USA); and avidin-rhodamin (Vector) and streptavidin-DyLight488 from ThermoScientific (Massachusetts, USA). 300 µm sections were gradually dehydrated in ethanol (Sigma) followed by clearing with BABB (Benzyl alcohol benzyl benzoate). Images were acquired using a LSM710 confocal microscope (Zeiss, Jena, Germany) equipped with a 5x (0,16 NA, Zeiss), 10x (0,30 NA, Zeiss), 20x (0,80 NA, Zeiss) and a 40x (1,30 NA, Zeiss) objective. Image analysis was performed using LSM Image Browser.

Quantification of GC volume and GC T cell density

GC volume was calculated (in cubic microns) multiplying the PNA^+ area identified in the XY plane of z-stacks, acquired with a confocal microscope, by the depth of the structure (z plane). The area was calculated using the LSM image browser software. Cell density was obtained dividing the number of positive cells by the volume of the corresponding GC.

ELISA assay.

Isotype-specific antibody titers in the serum were determined by ELISA on 96-well plates (BD Falcon) coated with 50 µg OVA (grade V, Sigma) in carbonate buffer pH=9.6 + N_3Na 0.01%. Nonspecific binding was blocked with 1% BSA (AMRESCO, Inc.) in PBST. Sera were diluted 1:50 or 1:100 in PBST, and threefold serial dilutions of serum samples were incubated. Negative controls (normal mouse serum diluted 1:100) were included in all assays. The plates were washed and 50 µl isotype-specific goat anti-mouse-horseradish peroxidase conjugates (SouthernBiotech) was added. ELISA's were developed with ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate (Sigma-Aldrich). The optical density (OD) at 405 nm was determined after 4, 5, and 6 min with an automatic ELISA plate reader (Envision Multilabel Reader 2104, Perkin Elmer) and the highest values were recorded.

Statistical analysis.

Statistical significance was determined using the two-tailed non-parametric Mann-Whitney test and P values <0.05 were deemed significant (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

4.3 Results

4.3.1 Co-development of the GCR and GC T cells

To analyze GC T cells, Balb/c mice were immunized with Ovalbumin (OVA) to trigger a GCR and sacrificed at days 0, 4, 12 and 16 in order to evaluate the initiation, peak and decline of GCR. mLN were collected and 20 µm sections were stained with PNA and anti-CD3 to identify GCs and T cells. After immunization the GCR peaked at about day 12 as measured by the average GC volume using confocal microscopy (Figure 1A, B) and by day 16 already declined to baseline level.

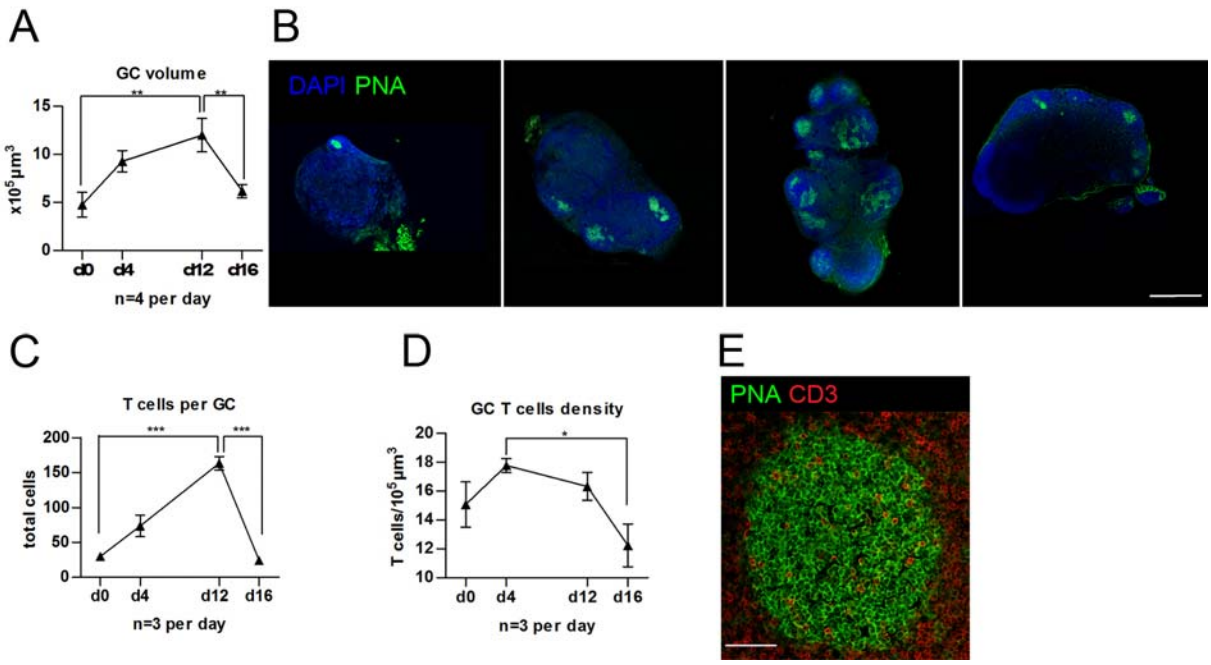

Figure 1. GC T cell density reaches maximum at day 4 of GCR. Balb/c mice were immunized with 20 µg OVA-alum i.p. and sacrificed at the indicated time points, when cryosections of mLN were analyzed to follow the GCR. (A) Average GC volume. GCs were identified by PNA staining and quantified by confocal microscopy. (B) Representative stainings from non-immunized mice, and mice sacrificed on days 4, 12 and 16 post immunization. Bar: 500 µm. (C) Total number of T cells and (D) density of GC T cells during a GCR as analyzed by confocal microscopy. GC T cells were identified as $CD3^+$ cells within the PNA area. (E) Representative staining from a LN on day 12 after immunization. Bar: 50 µm. Data are representative of two independent experiments. * P<0.05, ** P<0.01, *** P<0.001 (Mann-Whitney non-parametric , two-tailed test).

The quantification of total T cells within the GCs revealed that the kinetics of T cell numbers parallels that of the global GCR (Figure 1C). However, since GC volume increases during GCR, we calculated the density of T cells within the GC (i.e. number of T cells per unit of GC volume) and found that the T cell density was highest at day 4 post-immunization (Figure 1D).

4.3.2 Foxp3$^+$ GC T cell concentration increases during GCR

As illustrated in Figure 2A and already described before (chapter 3), Foxp3$^+$ cells were readily detectable within GCs of mLNs. After observing changes in the GC T cell density during the GCR, we investigated, if GC Foxp3$^+$ T cells would also change in numbers during the GCR.

We found that, while total GC T cells have the highest density at day 4 of GCR (Figure 1D), the density of Foxp3$^+$ cells increases steadily during the GCR up to day 12 and day 16 (Figure 2B), at a point when the GC reaches its maximum and starts to contract (Figure 1A, B). We confirmed the reliability of those data by analyzing non-consecutive sections from the same LN leading to similar results (Figure 2C).

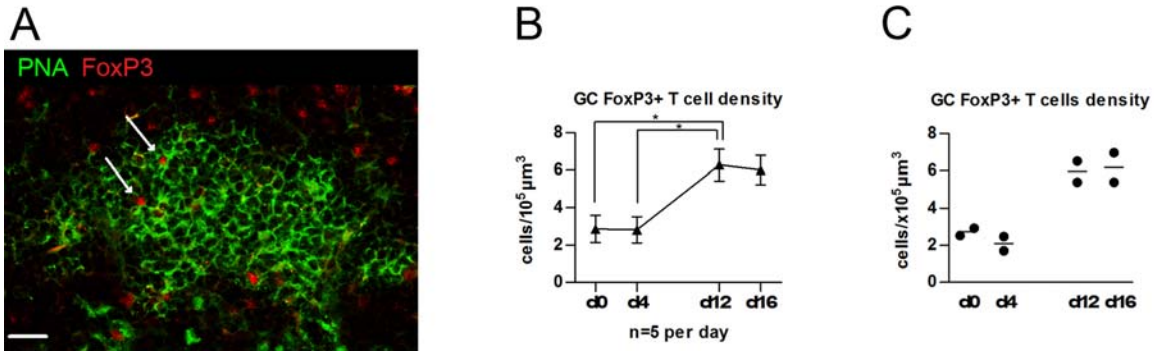

Figure 2. GC Foxp3$^+$ cell concentration reaches maximum at day 12 of GCR. Balb/c mice were immunized with 20 µg OVA-alum i.p. and sacrificed at the indicated time points. (A) Cryosections of mLN were stained with PNA and anti-Foxp3 to identify Foxp3$^+$ cells within the GC. Bar: 20 µm. (B) The density of Foxp3$^+$ T cells within the GC during the course of a GCR was quantified by confocal microscopy. The density corresponds to the total number of Foxp3$^+$ cells within the PNA$^+$ area divided by the measured GC volume. (C) The density of Foxp3$^+$ T cells within the GC during the course of a GCR from 2 different non-consecutive sections of the same LN, each dot represents the data from one section. Data are representative of two independent experiments. * P<0.05 (Mann-Whitney non-parametric , two-tailed test).

4.3.3 GC Foxp3$^+$ T cells are highly proliferative

We next assessed the proliferative behavior of GC Foxp3$^+$ T cells by quantifying the number of these cells positive for the proliferation marker Ki67. As shown in Figure 3, the frequency of proliferating (Ki67$^+$) Foxp3$^+$ cells within the GC is greater than the frequency of proliferating Foxp3$^+$ cells outside the GC. In addition, there is a significant increase of proliferation at day 12 post-immunization – a time that, as discussed above, correlates with an increase in the frequency of Foxp3$^+$ cells within the GC. It is noteworthy that at day 4 post-immunization the proliferation of Foxp3$^+$ cells increases significantly outside the GC. On the contrary, the frequency of proliferating cells within total GC T cells does not change during the GCR.

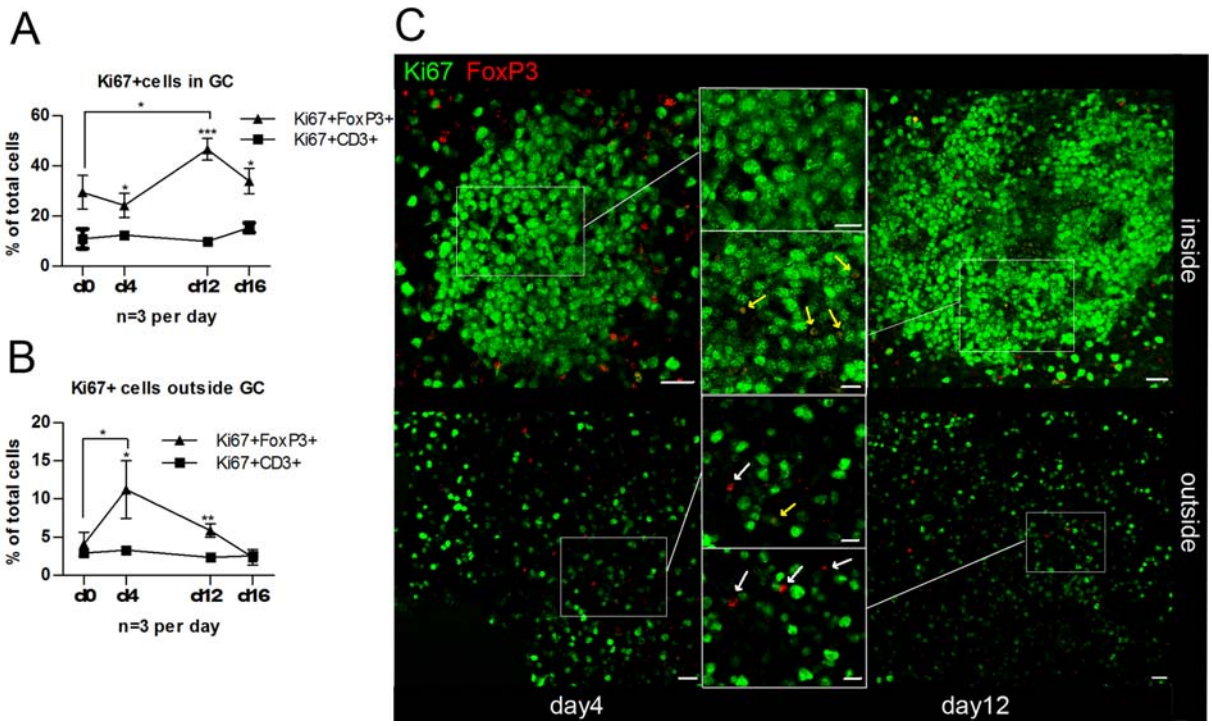

Figure 3. GC Foxp3$^+$ T cells show an increased proliferation during the GCR. Balb/c mice were immunized with OVA-alum as described above. The frequency of proliferating T cells was quantified by confocal microscopy based on Ki67 expression. (A) Proliferating GC Foxp3$^+$ cells and GC T cells were identified as, respectively, Ki67$^+$Foxp3$^+$ cells and Ki67$^+$CD3$^+$ cells within the PNA area, and are depicted as % of the total Foxp3$^+$ or total CD3$^+$ cells identified in the GC. (B) A similar analysis was performed for T cells outside the GC. (C) Representative stainings of proliferation of GC T cells. Bar: 20 µm and 10 µm for the zoom in. Data are representative of two independent experiments. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 (Mann-Whitney non-parametric, two-tailed test).

4.3.4 Absence of Foxp3$^+$ T cells enhances the magnitude of the GCR

After observing an increased frequency of Foxp3$^+$ GC T cells at day 12 post immunization, we assessed the functional significance of those cells for the regulation of GCR. For this purpose we used TCRα$^{-/-}$ mice, which lack α/β T cells and hence also lack Treg cells, and reconstituted those mice with OVA-specific CD4 T cells from OT2.Rag2$^{-/-}$ mice (that are also devoid of Treg cells). The OT2 cells were transferred alone, or together with sorted Treg cells from Foxp3^{gfp} knock-in reporter mice. The recipient mice were immunized with OVA, and GCR was followed at different time points, as described above.

Since TCRα$^{-/-}$ mice are known to show an impaired ability to develop GCs due to their lack of α/β T cells (Lindroth et al., 2002) (Figure 4A), we first ensured that TCRα$^{-/-}$ mice could develop GCs after adoptive cell transfer of antigen-specific T cells. Lymph node sections from TCRα$^{-/-}$ mice transferred with OT2 cells and immunized with OVA-alum were analyzed for the presence of GCs by staining with PNA and anti-IgM. These experiments revealed the ability of transferred OT2 cells to support a GCR, with a relatively high magnitude (Figure 4B, C). However, co-transfer of Foxp3$^+$ Treg cells together with OT2 cells, reduced significantly the

magnitude of the GCR (Figure 4C). Those results were supported by the increased percentage of GC B cells ($CD19^+GL7^+FAS^+$) in the mLN that we observed by FACS analysis in mice without Treg cells (Figure 4D). To study, if the effect of Treg cells on the GCR extends als to impact the production of class switched antibodies, we analyzed the serum titers of anti-OVA antibodies.

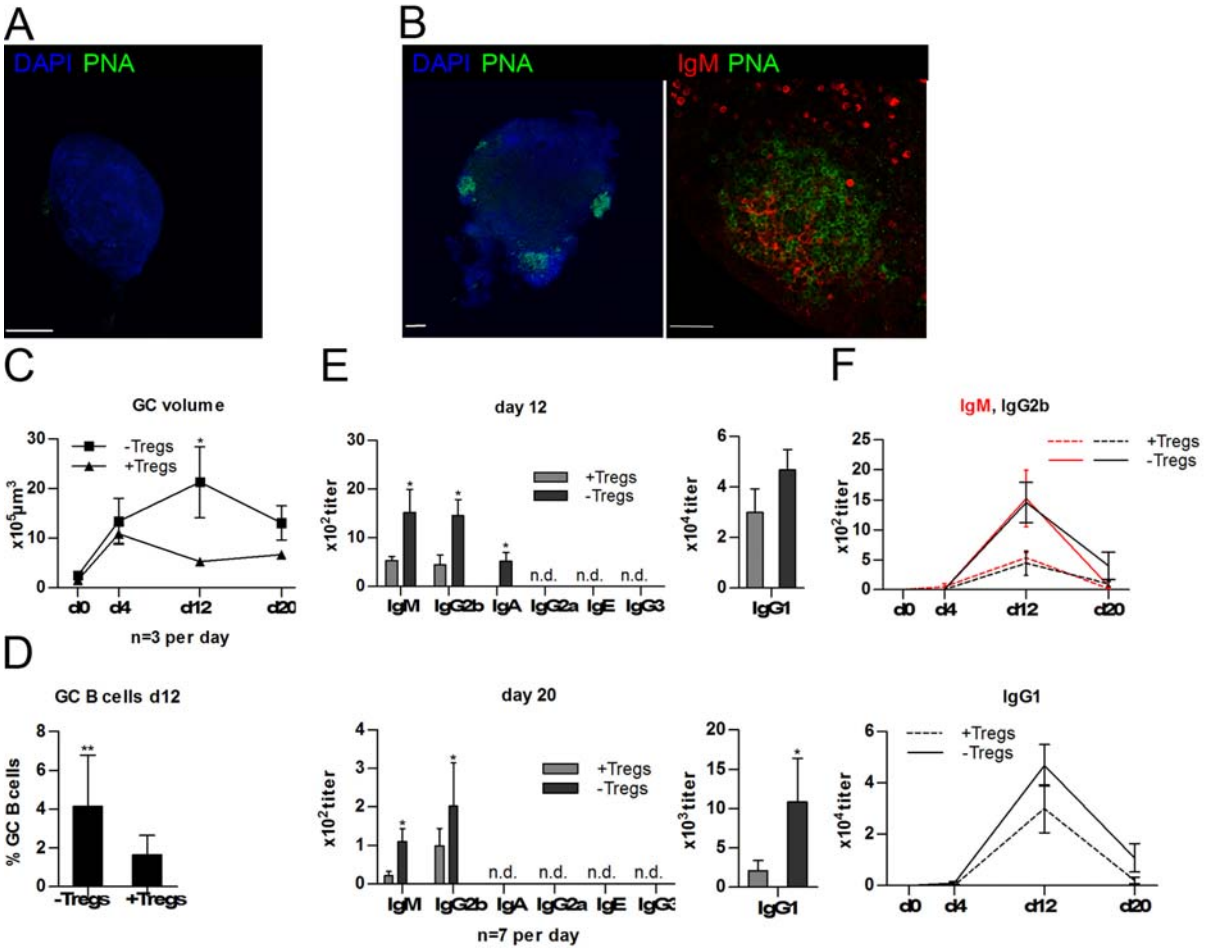

Figure 4. Lack of $Foxp3^+$ T cells enhances the magnitude of GCR in vivo. (A) MLN of $TCR\alpha^{-/-}$ mouse without reconstitution of αβ T cells immunized with OVA-alum at day 12 post-immunization. Sections were stained with PNA and DAPI to reveal GCs and cell nuclei (bar: 500 µm). (B) $TCR\alpha^{-/-}$ mice were adoptively transferred with 10^4 OVA-specific T cells from $OT2.Rag2^{-/-}$ mice together with or without equal numbers of sorted $Foxp3^{gfp+}$ T cells and immunized with OVA-alum. The micrographs show GCs in mice that did not receive $Foxp3^+$ T cells at day 12 post-immunization. left bar: 200 µm, right bar: 50 µm. (C) Quantification of the GC volume from mLN by confocal microscopy. GCs were identified as a PNA^+ region within the follicle marked by IgM. (D) Quantification of GC B cells at day 12 post-immunization by flow cytometry. (E,F) Serum titers of OVA-specific immunoglobulin of different classes were determined by ELISA at different times after immunization (n.d. – not detected). Data are representative of two independent experiments. * $P<0.05$, ** $P<0.01$ (Mann-Whitney nonparametric, two-tailed test).

Consistently with our above data, we found that the adoptive transfer of $Foxp3^+$ Treg cells had an impact on antibody production, leading to lower serum titers of secreted OVA-specific IgG1, IgA, IgG2b and IgM (Figure 4E,F).

All antibody titers in both groups were reduced approximately one log by day 20, but with mice transferred with Treg cells still showing lower serum titers compared to mice without Treg cells (Figure 5F). Collectively, these data suggest that Treg cells have an important regulatory effect on the magnitude of the GCR and antibody production after immunization.

4.3.5 Foxp3^{+}GC T cells regulate the magnitude of GCR

Given the fact that the initiation of the GCR takes place outside of the B cell follicle and is also highly dependent on the number of Th cells available for B cell help, it was possible, that the observed regulation was an early process acting on extra-follicular Th cells before the start of the GC. In order to address whether Foxp3^{+}Treg cells required access to the follicle to exert their suppressive function, we took advantage of CXCR5$^{-/-}$ mice. T cells from these mice cannot access the follicle, as they lack the chemokine receptor that enables them to follow CXCR5 expressed by stromal cells in the B cell follicle (Hardtke et al., 2005). Then we repeated the adoptive cell experiments using Treg cells not able to access the follicle. Importantly, as already demonstrated by Hardtke et al using confocal microscopy, CXCR5$^{-/-}$ mice lack T cells in the B cell follicle (Hardtke et al., 2005). Moreover flow cytometry analysis of the mLN of immunized CXCR5$^{-/-}$ mice showed that those mice lack the typical profile of T_{FH} cells (Figure 5A).

TCRα$^{-/-}$ mice were adoptively transferred with a 1:1 mix of OT2 cells and CD4^{+}CD25^{bright} Treg cells (that we confirmed were predominantly Foxp3^{+}) from Foxp3^{gfp} or CXCR5$^{-/-}$ mice. As expected, transferred CXCR5$^{-/-}$Treg cells were not able to enter the GC (Figure 5D), as well as the B cell follicle (data not shown). We found TCRα$^{-/-}$ mice transferred with CXCR5$^{-/-}$Tregs showed a GCR with a higher magnitude (Figure 5B) comparable to mice that did not received Treg cells (Figure 4C). Consistently, mice transferred with CXCR5$^{-/-}$ Treg cells also showed increased percentages of GL7^{+}FAS^{+} GC B cells in the mLN at day 12 of GCR (Figure 5C).

Although the number of effector and regulatory T cells in our transfer system was smaller compared to wildtype conditions, the ratio total CD4^{+} T cells:Treg cells observed in GCs was maintained (Figure 5E, F).

We confirmed the above data, by adotptively transferring TCRα$^{-/-}$ mice with a 1:1 mix of OT2 cells and CXCR5^{-}PD1^{-}CD4^{+}CD25^{bright} Treg cells from Foxp3^{gfp} or CXCR5$^{-/-}$ mice. As shown in chapter 3 non-follicular Treg cells are able to acquire *in vivo* the follicular phenotype. Furthermore, the transferred wildtype Treg cells were able to control the GCR (Figure 5G), as TCRα$^{-/-}$ mice transferred with CXCR5$^{-/-}$Tregs showed at day 12 after immunization a higher GCR. Consistently, we found that the adoptive transfer of Foxp3^{+} Treg cells had an impact on antibody production, leading to lower serum titers of secreted OVA-specific IgG1, IgG2b and IgM (Figure 5H).

Taken together these data demonstrate a physiological role for follicular Foxp3^{+} T cells during the GCR and for the production of antibodies.

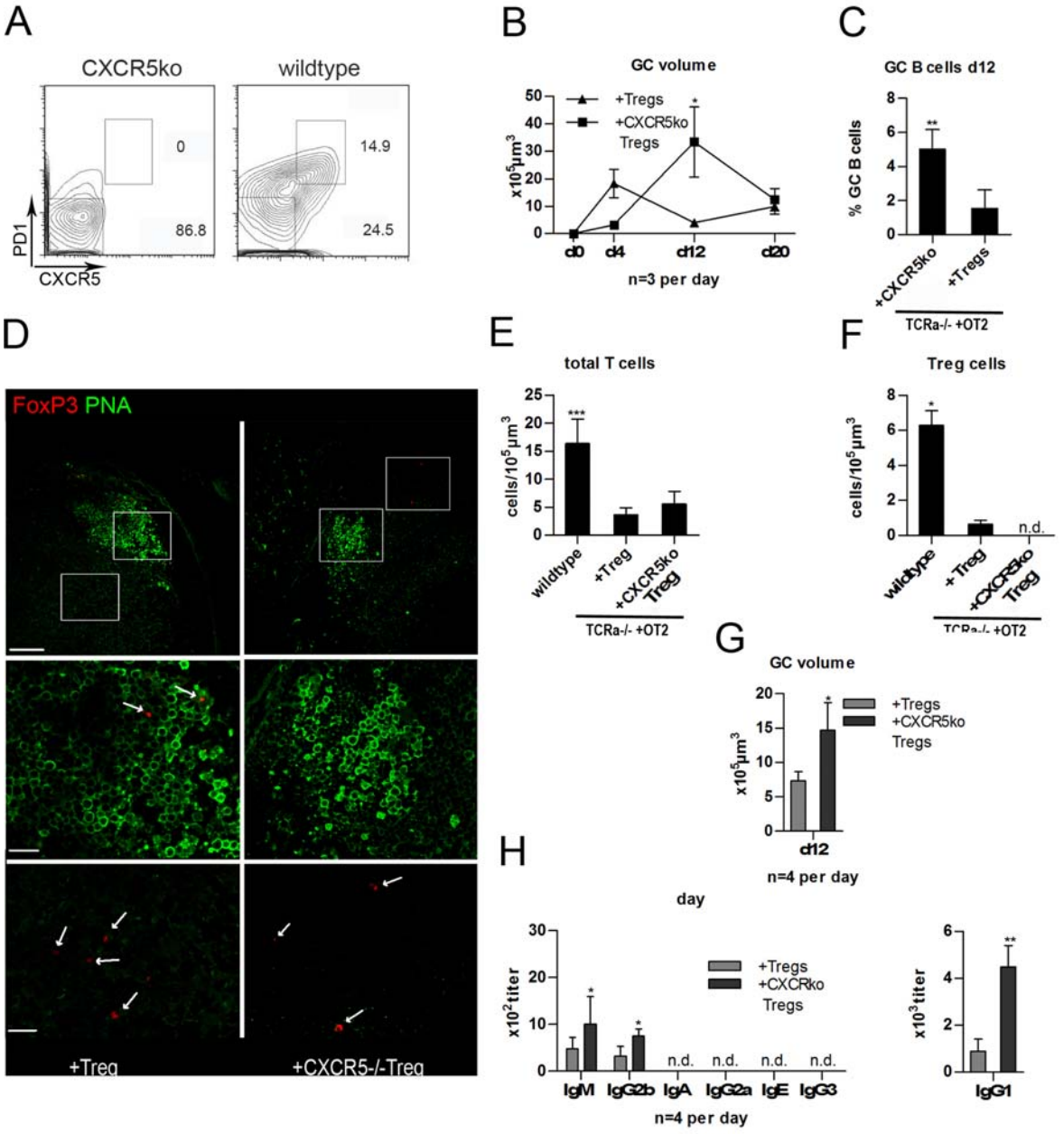

Figure 5. Follicular Foxp3$^+$ T cells regulate the magnitude of GCR in vivo. (A) CXCR5$^{-/-}$ mice or C57Bl/6 mice were immunized with OVA-alum and T_{FH} cells of mLN analyzed by flow cytometry at day 12 post-immunization. (B) TCRα$^{-/-}$ mice were adoptively transferred with 10^4 OVA-specific T cells from OT2.Rag2$^{-/-}$ mice together with equal numbers of sorted CD4$^+$CD25^{bright} from Foxp3^{gfp} or CXCR5$^{-/-}$ mice and immunized with OVA-alum. The GC volume in 50 µm sections of mLNs was quantified as detailed above. (C) GL7$^+$FAS$^+$ GC B cells in the mLN were quantified at day 12 of GCR by flow cytometry. (D) Representative micrographs from recipient mice, that received wildtype Treg or CXCR5$^{-/-}$ Treg cells at day 12 of GCR. GC area is stained with PNA, Treg cells with anti-Foxp3. Bar upper raw 100 µm, lower raws 20 µm). (E) Quantification of total T cells and (F) Foxp3$^+$ Treg cells within GCs using confocal microscopy (n.d. – not detected). (G) TCRα$^{-/-}$ mice were adoptively transferred with 10^4 OVA-specific T cells from OT2.Rag2$^{-/-}$ mice together with equal numbers of sorted CXCR5$^-$PD1$^-$CD4$^+$CD25^{bright} from Foxp3^{gfp} or CXCR5$^{-/-}$ mice and immunized with OVA-alum. The GC volume in 50 µm sections of mLNs was quantified as detailed above at day 12 after immunization. (H) Serum titers of OVA-specific immunoglobulin of different classes were determined by ELISA at day 12 after immunization (n.d. – not detected). Data are representative of two independent experiments. * P<0.05, ** P<0.01, *** P<0.001 (Mann-Whitney nonparametric, two-tailed test).

4.4 Discussion

The regulation of the GCR is not yet fully understood and represents a topic of current interest. Due to the absence of experimental data, there are various mathematical models trying to explain the regulation and termination of the GCR. One hypothesis, proposed that it was due to an increased impairment of the engagement of B cell receptors and antigen through either antigen consumption (Kesmir and De Boer, 1999) or antigen masking (Tarlinton and Smith, 2000). Both of these concepts assume that GC termination is regulated by the amount of antigen presented within the GC by FDCs. Another hypothesis suggests GC termination is caused by increased differentiation of GC B cells towards memory and plasma cells due to signals from FDCs (Moreira and Faro, 2006). A third hypothesis proposes that signaling from T cells is the responsible for GC termination (Moreira and Faro, 2006).

After observing an increase in follicular Foxp3$^+$ T cell numbers in the GC towards the end of the GCR, we hypothesized a scenario whereby this cell population plays a role in controlling the outcome of a GCR.

Our data show that follicular Foxp3$^+$ T cells have a strong impact on the magnitude of the GCR, as well as the resulting production of antigen-specific antibodies. The lack of Treg cells in adoptive cell transfer experiments profoundly modified the outcome of the GCR, as those mice showed larger GCs at day 12, and greater production of different classes of Ig, than in the presence of Treg cells. Moreover, using Treg cells isolated from CXCR5$^{-/-}$ mice, we created a system that has Treg cells but those are unable to gain access to B cell follicles. The data obtained in that system show that Treg cells require access to the follicle to control the GCR, in other words a follicular Treg cell population is required for plain control of the GCR. Although our data do not prove unequivocally a role for follicular Treg cells in the termination of the GCR, we clearly show follicular Treg cells affect the magnitude of this reaction. Moreover we were able to show that follicular Treg cells have *in vivo* direct impact on the GC B cell population. Further studies are necessary to show whether this regulation operates by controlling effector T_{FH} cells, and by that indirectly affecting GC B cells, if that regulation acts directly on GC B cells, or if it affects both T_{FH} and GC B cells.

GC B cells greatly increase in numbers during the GCR due to massive proliferation (Allen et al., 2007a). We found that GC T cells also increase in numbers during this process, being even more conspicuous the increase of the FoxP3$^+$ GC T cell subpopulation. Total GC T cells reached their highest density at day 4 of GCR and declined afterwards. However this did not seem to be caused by increased proliferation. Therefore, it is likely that already existing T_{FH} cells or the novo differentiated T_{FH} cells migrate into the GC. On the other hand, Foxp3$^+$ GC T cells reached their highest concentration between days 12 and 16 of the GCR, at a time when the total GC T cell density had already decreased, and the Foxp3$^+$cells showed an increased proliferation at day 12. Moreover when we measured the proliferation of Foxp3$^+$ GC T cells outside the GC we found that they had an increased proliferation at day 4. We therefore propose that follicular Foxp3$^+$ T cells start proliferating at the initiation of the GCR, followed by a migration into the developing GC. In the GC the follicular Foxp3$^+$ T cells keep

proliferating, which shifts the balance between effector cells and regulatory cells and ultimately leads to the regulation of GCR. It was already shown, that conventional Foxp3$^+$ Treg cells are able to suppress B cells (Lim et al., 2005). We observed, that follicular Foxp3$^+$ T cells in the GC are still at high concentrations towards the end of the GCR, suggesting that they could not only control GC T cells, but could be also involved in controlling GC B cell numbers. Moreover, using an in vivo system that lacks follicular Foxp3$^+$ T cells we were able to show that the GC B cell population increased drastically compared to mice that contained follicular Foxp3$^+$ T cells. However, further work is necessary to show, if this suppression is achieved by Foxp3$^+$ GC T cells acting directly on GC B cells or through decreasing numbers of GC T cells and consequent lack of B cell help.

GC deregulation or malfunctions are linked to the onset of severe diseases, as cancer and autoimmunity (Vinuesa and Cook, 2001). Taken together, our data show that follicular T cells include a new regulatory subpopulation, which by controlling the GCR may ultimately impact on preventing such pathologies.

5. General discussion

The generation of a humoral immune response to foreign TD antigens requires the rapid expansion of antigen-specific CD4 T cells after their recruitment to the SLO where they collaborate with B cells. In the T-B border they provide help to antigen-specific B cells, leading to a fast generation of non-somatically mutated short lived plasma cells by providing help to B cells (Jacob et al., 1991a; Liu et al., 1991b). In B cell follicles CD4 T cells initiate the comparatively slow GCR, which consequently will give rise to long-lived high-affinity plasma and memory B cells (Ho et al., 1986; Slifka et al., 1998). Additionally, following re-exposure to antigen, primed memory CD4 T cells supply help to both memory and naive B cells resulting in faster and more efficient secondary immune responses.

The molecular, as well as cellular interactions that drive T cells to help B cells during their activation are still to be fully elucidated. Cell transfer of *in vitro* differentiated Th2 cells showed that they are more efficient inducers of GCs than Th1 cells (Randolph et al., 1999; Secord et al., 1996). This was associated with the preferential migration of Th2 cells to the T/B border (Randolph et al., 1999). The CD28 costimulatory signal, provided by DCs to T cells, is crucial for GC development (Ferguson et al., 1996; Lane et al., 1994). Activation of CD4 T cells through CD28 leads to upregulation of OX40 (Walker et al., 1999), which is absent on naïve T cells (Mallett et al., 1990). OX40 expression enables activated CD4 T cells to receive signals through OX40L expressed by DCs (Brocker et al., 1999; Ohshima et al., 1997). *In vitro* and *in vivo*, OX40 signals can upregulate expression of the chemokine receptor CXCR5 on T cells, which allows them to migrate towards B cell follicles (Forster et al., 1996) where the CXCR5 ligand CXCL13 is expressed by stromal cells (Gunn et al., 1998).

CXCR5 together with PD-1 expression is a hallmark of T_{FH} cells, which are known, to be important for the GCR (Fazilleau et al., 2009). Therefore it is plausible that sequential signals from DCs through CD28 and then OX40 are components of the signaling pathway that directs T cells to migrate to and interact with B cells. From this point of view, OX40L could be an important signaling molecule during humoral responses to foreign antigens.

We investigated the effect of OX40-OX40L blockade in the development of allergic airways disease by utilizing a non-depleting anti-OX40L mAb.

Sensitization and subsequent airway challenge of BALB/c mice with OVA resulted in a typical asthma-like inflammation of the airways characterized by eosinophilia in the BAL, high antigen-specific IgE and IgG1 levels in the serum, high levels of Th2 cytokines (IL- 4, IL-5, and IL-13) in the lung, as well as goblet cell hyperplasia and AHR. We demonstrated that OX40L blockade can drastically reduce the manifestations of allergic airways disease. We documented a strong reduction in airways eosinophilia, as well as in production of IL-4, IL-5 and IL-13 in the lungs and in serum levels of IgE and IgG1.

We furthermore confirmed by histology, that anti-OX40L treated mice had no inflammatory lung infiltrates. Measurement of respiratory mechanics in response to inhaled methacholine

could confirm that the airways of mice treated with anti-OX40L were physiologically similar to healthy unmanipulated controls, showing no AHR.

These results are in agreement with a previous report demonstrating that the development of asthmatic manifestations, like eosinophilia, mucus overproduction, and AHR, was abrogated in OX40-deficient mice. Those mice also showed severely impaired Th2 responses after sensitization and airway challenge with OVA (Jember et al., 2001), which was subsequently reproduced in OX40L knockout mice (Arestides et al., 2002).

In asthmatic patients there is an accumulation of Th2 cells in the airways, producing the cytokines IL-4, IL-5 and IL-13 (Mazzarella et al., 2000; Robinson et al., 1992). The crucial role of Th2 cytokines in the development of asthma was assessed in murine models using IL-4, IL-5, and IL-13 deficient mice, demonstrating that IL-4 is responsible for IgE overproduction, IL-5 for eosinophilia and IL-13 for mucus overproduction and AHR (Foster et al., 1996; Kuhn et al., 1991; Wills-Karp et al., 1998). As a consequence, the abrogation of IgE production, eosinophilia, and goblet hyperplasia, as well as AHR by OX40L blockade is likely to be due to the suppression of IL-4, IL-5, and IL-13 (Wills-Karp, 1999).

To investigate whether OX40L blockade might represent a clinically relevant treatment strategy in patients with already established asthma, we first sensitized animals with OVA and waited one month before re-sensitizing the animals during treatment with anti-OX40L mAb. In this case, the treatment was not effective in pre-sensitized mice. Our results differ from a previous report by Salek-Ardakani et al who described, that OX40L blockade efficiently prevented the hallmarks of allergic asthma in pre-sensitized mice (Salek-Ardakani et al., 2003). The conflicting results may be due to differences in the study design and to the use of a different mAb. Whereas our anti-OX40L mAb is IgG1, Salek-Ardakani et al used an IgG2b mAb. It is well known that mAbs with different isotypes can have distinct effects, when used for therapeutic purposes (Seshasayee et al., 2007). In addition, in the study by Salek-Ardakani et al, mice were sensitized at day 0 and then intranasally challenged at day 24. On the contrary, in our study, mice were immunized twice at days 1 and 14 and then challenged at day 21, 22 and 23. This protocol leads to a stronger and more prolonged effector response. In addition, Salek-Ardakani et al blocked OX40-OX40L signalling at the time of airways exposure to the allergen, a time when it was already shown using a protocol similar to ours, that OX40L blockade has no effect (Hoshino et al., 2003). Taken together these data support the notion that the protocol used by Salek-Ardakani et al leads to a milder allergic response, which is more susceptible to manipulation.

Intestinal inflammation is associated with an increase in $OX40^+$ and $OX40L^+$ cells in the gut (Malmstrom et al., 2001; Souza et al., 1999) and blockade of OX40-OX40L signalling inhibits pathologic inflammation in mouse models of colitis (Malmstrom et al., 2001; Takeda et al., 2004). In these models of autoimmunity OX40L blockade appears to lead to reduction of disease severity due to reestablishment of immunological tolerance. Therefore, we investigated, whether OX40L blockade is able to induce tolerance in a model of allergic airways disease. We found that anti-OX40L treatment did not prevent allergic airways

disease when the mice were treated with anti-OX40L mAb in advance of a second sensitization with the same antigen.

In the last two decades a maior role of regulatory T cells in immune tolerance was established by many investigators (Wing and Sakaguchi, 2010). Nevertheless, there are many conflicting results from *in vitro* and *in vivo* studies about the role of OX40 signaling in Treg cell-mediated immune tolerance. OX40 signaling has been reported to be neutral or can promote or even to inhibit Treg cell mediated suppression (Golovina et al., 2008; Hippen et al., 2008; Piconese et al., 2008; Takeda et al., 2004; Valzasina et al., 2005). However, a recent study by the group of Fiona Powrie showed that OX40 expression by Treg cells was crucial for Treg cell accumulation in the lymphoid organs and their contribution to immune tolerance (Griseri et al., 2010).

Based upon these observations, it is plausible that OX40L blockade can protect mice from immediate inflammatory reaction, but due to interference with Treg function, long-term protection cannot be acomplished.

T_{FH} cells are a crucial subpopulation of CD4+ T cells to mount a humoral immune response against TD antigens. They provide help to GC B cells and allow the formation of long-lived plasma cells and the development of memory B cells with high affinity. In mice, the migration of primed T cells into follicles and their differentiation into T_{FH} cells seems to depend on sequential stimuli that involve DCs, $CD4^+CD3^-$ LTi cells, B cells and signals from follicular stromal cells. In the T cell zone T cell priming by DCs, through CD28-mediated co-stimulation, results in upregulation of OX40 and CXCR5 expression by T cells (Walker et al., 1999). This initial upregulation of CXCR5 expression, combined with downregulation of CCR7 expression, causes T cells to migrate to the T-B border. In this location, activated antigen-specific $CD4^+$ T cells interact with B cells and LTi cells (Ansel et al., 1999; Garside et al., 1998; Kim et al., 2003). The LTi cells that are present at the T-B border and within follicles, provide signals through OX40 and CD30 molecules at the surface of activated $CD4^+$ T cells, which contributes to the final location of these T cells, maintenance of GCRs and generation of B-cell and $CD4^+$ T-cell memory responses (Gaspal et al., 2005; Kim et al., 2003).

Furthermore signals induced through OX40L can influence the number of T_{FH} cells. Naive T cells that are stimulated by DCs transfected with OX40L upregulate CXCR5 expression (Flynn et al., 1998), and mice that overexpress OX40L at the surface of DCs have increased numbers of T cells in the follicles (Brocker et al., 1999). However, primary T cells fail to migrate into the follicles of immunized OX40-deficient mice or mice with compromised OX40 signalling (Fillatreau and Gray, 2003), and a combined deficiency in OX40 and CD30 severely impairs memory antibody responses (Gaspal et al., 2005; Kim et al., 2003). Finally, survival of mouse memory T_{FH} cells, necessary for induction of secondary antibody responses, has been shown to require IL-7 secreted by lymphoid stromal cells. IL-7 partly exerts its effects through the induction of OX40 expression (Gaspal et al., 2005).

Regardless of an improved understanding of the molecular program that drives T_{FH} cell formation, it has been shown that CD4 T cells in B cell follicles are heterogeneous (Vinuesa

et al., 2005; Yu et al., 2009a). In this study we have contributed to elucidate that heterogeneity by identifying a new Foxp3$^+$ T cell subpopulation within follicular (CXCR5$^+$PD-1$^+$) CD4 T cells, that exerts suppressive functions.

FoxP3$^+$ Treg cells are specialized in suppressing various types of effector cells. This property makes them vital to maintain immune tolerance and prevent many inflammatory diseases (Sakaguchi et al., 2008). Many homing molecules like CD62L, CCR7, CCR6, CXCR4 involved in the control of migration during inflammatory responses, have been identified on Treg cells in different immunological settings (Campbell and Koch, 2011).

Recently, it has become clear that Treg cells can be divided into several distinct subsets, displaying unique functional and homeostatic properties, that work side by side to maintain normal immune homeostasis (Feuerer et al., 2009).

We have found that follicular Foxp3$^+$ CD4$^+$ T cells expressed CD25, GITR and CD103 which are characteristically expressed by conventional Treg cells (Lehmann et al., 2002; Sakaguchi et al., 1995; Shimizu et al., 2002). Consequently follicular Foxp3$^+$ CD4$^+$ T cells could represent an additional regulatory subset. CD103 was shown to be important for homing of Treg cells to the gut and skin (Weiner et al., 2011). The high expression of CD103 by follicular Foxp3$^+$ T cells suggests that besides CXCR5, CD103 could represent another important homing molecule for those cells.

Bcl-6 is the the master transcription factor for the T_{FH} lineage, by switching on a gene repressor program that involves the suppression of key transcriptional regulators of other T helper cell lineages, namely Tbet (Th1) and RORγt (Th17). Yet a suppression of the Treg lineage key transcription factor Foxp3 was never observed (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009b). Moreover, we have shown that, in contrast to extra-follicular Foxp3$^+$ Treg cells, follicular Foxp3$^+$ CD4$^+$ T cells express the transcription factor Bcl-6. Due to this unique phenotype we name these cells $T_F$reg cells.

Lim et al found that human tonsils contain a CD69$^-$CD4$^+$CD25$^+$ T cell population that was able to suppress in vitro antibody production by CD19$^+$ B cells, but without the potential to migrate to the B cell follicles. They also studied in the same report a CD25$^+$CD69$^+$ population with no regulatory function, but with the potential to migrate to the B cell follicles (Lim et al., 2004). Our data show that $T_F$reg cells express high amounts of CD69 and display suppressive capacities comparable to non-follicular Treg cells. In addition the presence of follicular Treg cells in the B cell follicle could be confirmed by confocal microscopy. The discrepancy between the two studies is likely due to the fact that Lim et al studied human tonsils and our study was based on murine mesenteric lymph nodes. Future studies will have to confirm the existence and analyze the phenotype of follicular Foxp3$^+$ regulatory T cells in human subjects.

In primary immune responses the GCR peaks characteristically at day 12 post-immunization and declines afterwards. We show here that total CD4$^+$ T cells reached their highest density at day 4 of GCR, but without detectable increased proliferation. For this reason, it is likely that already existing T_{FH} cells migrate into the GC. In contrast, $T_F$reg cells had their maximum

density from day 12 of GCR onwards, when total GC $CD4^+$ T cell density already decreases and show a significantly augmented proliferation at days 12 and 16. Furthermore when we assessed the proliferation kinetics of extra-follicular $Foxp3^+$ T cells we found they had a peak of proliferation at day 4. Therefore it is plausible that $T_F$reg precursor cells start proliferating at the beginning of the GCR, followed by their migration into, and differentiation within, developing GCs. Within GCs $T_F$reg cells increase their proliferation when T_{FH} cells are at their height. This causes a change in the effector/regulatory cell ratio, thus possibly leading to a tighter control of GC Tcells.

TGFβ is crucial for the peripheral conversion of $Foxp3^-$ T cells into $Foxp3^+$ Treg cells (Chen et al., 2003; Oliveira et al., 2011a). Surprisingly, we could show that adding TGFβ to *in vitro* cultures of $CD4^+Foxp3^-$ non-follicular T cells, but not to cultures of $CD4^+Foxp3^-$ follicular T cells, induces Foxp3 expression. Moreover, *in vivo* studies confirmed that T_{FH} cells are unlikely to acquire Foxp3 expression. However, non-follicular Treg cells acquired *in vivo* a follicular phenotype during a secondary immune response, thus pointing to an extra-follicular Treg cell as precursor for $T_F$reg cells.

The regulation of the GCR is of great current interest, but due to lack of experimental data it is still not fully understood. At present there are various mathematical models trying to explain the termination and regulation of the GC. Two models explain the termination of the GCR with an reduced engagement of B cell receptors and antigen through either antigen consumption (Kesmir and De Boer, 1999) or antigen masking (Tarlinton and Smith, 2000). Both of these ideas presume that GC termination is regulated by the quantity of available antigen presented within the GC by FDCs. Another theory explains GC termination by amplified differentiation of GC B cells towards memory and plasma cells due to signals from increasingly differentiated FDCs (Moreira and Faro, 2006). A third hypothesis proposes that signaling from further maturated T_{FH} cells are accountable for GC termination (Moreira and Faro, 2006). Our finding that GC T cells comprise the new regulatory subpopulation of $T_F$reg cells prompted us to investigate, if these cells influence the GCR and ultimately the outcome of a humoral immune response.

In support of this hypothesis, we were able to demonstrate, that follicular $Foxp3^+$ T cells have a strong impact in the extent of the GCR, as well as the resulting production of specific antibodies. Lack of Treg cells in our transfer system greatly modified the outcome of the GCR, as those mice showed larger GCs at the GC maximum, and greater production of different classes of Ig, than in the presence of Treg cells. Furthermore, using $CXCR5^{-/-}$ Treg cells we demonstrated that lack of $T_F$reg cells led to increased GC B cell numbers as well as an increased size of the developing GCs. These data suggest that $T_F$reg cells have a direct impact on the quality of the produced antibodies.

Conventional $Foxp3^+$ Treg cells are capable of suppressing B cell responses (Lim et al., 2005). Our demonstration, that $T_F$reg are at high density at the end of the GCR, implicates that they might not only control GC T cells, but could also be involved in controlling GC B cells.

Although our data do not explicitly show a role for $T_F$reg cells in the termination of the GCR, they undoubtedly demonstrate that $T_F$reg cells influence the magnitude of this response. Additionally we were able to show that $T_F$reg cells have direct impact on the GC B cell population *in vivo*. Further studies are needed to show whether this regulation operates by controlling effector T_{FH} cells, and by that indirectly affecting GC B cells, or alternatively if regulation acts directly on GC B cells or even if it affects both T_{FH} and GC B cells simultaneously.

GC deregulation or malfunctions in the GC processes like CSR and affinity maturation are associated to the onset of severe diseases, like cancer and autoimmunity (Vinuesa and Cook, 2001). Taken together, our data show that $T_F$reg cells include a new regulatory subpopulation, which had a strong impact on the number of GC B cells and the quality of the produced Abs, thus $T_F$reg cells may ultimately impact the prevention of such pathologies.

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7. Appendix

Full-length article

Regulation of the germinal centre reaction by Foxp3^{+} follicular T cells

Running title: Foxp3^{+} follicular T cells.

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Abstract

Follicular helper T cells (T_{FH}) cells participate in humoral responses providing selection signals to germinal center B cells. Recently, expression of CXCR5, PD-1, and the transcription factor Bcl-6 has allowed the identification of T_{FH} cells. We found that a proportion of follicular T cells, with phenotypic characteristics of T_{FH} cells and expressing Foxp3, are recruited during the course of a germinal centre (GC) reaction. These Foxp3$^+$ cells derive from natural regulatory T cells. In order to establish the *in vivo* physiological importance of Foxp3$^+$ follicular T cells we used CXCR5-deficient Foxp3$^+$ cells, which do not have access to the follicular region. Adoptive cell transfers of CXCR5-deficient Foxp3$^+$ cells have shown that Foxp3$^+$ follicular T cells are important regulators of the GC reaction following immunization with a thymus-dependent antigen. Our *in vivo* data show that Foxp3$^+$ follicular T cells can limit the magnitude of the GC reaction and also the amount of secreted antigen-specific IgM, IgG1, IgG2b and IgA. Therefore, Foxp3$^+$ follicular T cells appear to combine characteristics of T_{FH} and regulatory T cells for the control of humoral immune responses.

Introduction

Germinal centers (GCs) are temporary structures in secondary lymphoid organs in which antigen (Ag)-specific B cells undergo extensive proliferation, class-switch recombination and somatic hypermutation (SHM), an unique process whereby they may acquire higher affinity for the antigen (1). Across several cell divisions, high affinity GC cells are selected and differentiate eventually into either memory cells or long-lived plasma cells, providing the basis of a process called affinity maturation of serum antibodies (Ab). Long-term humoral immunity, as it is achieved in many prophylactic vaccines, is provided by those plasma cells and memory B cells and is a critical component to protect the body during subsequent infection (2).

In the last 15 years an important body of information concerning the role of so-called follicular T helper (T_{FH}) cells in the GC reaction (GCR) has been obtained. This is a distinct subpopulation of $CD4^+$ T cells characterized by expressing high membrane levels of CXCR5 (the receptor of the follicular chemokine CXCL13, essential for follicular homing) and PD-1 (a key molecule for B cell survival and selection, presumably through interaction with PD-L2 (3) and high levels of the transcription factor Bcl-6 (4, 5). Productive interactions between GC B cells and T_{FH} cells are mediated by cognate interactions through T cell receptor and major histocompatibility complex class II/peptide interactions, CD40-CD40L and ICOS-ICOSL (6). When CD40-CD40L interactions are prevented, the on-going GCR stops and GCs dissolve within 24 h (6). Mice deficient in ICOS or ICOSL also have impaired GC formation and isotype switching (7). Triggering of the SHM process in GC B cells is also dependent on CD40-CD40L interactions (8) Immunized mice that lack T_{FH} cells show reduced numbers of germinal center B cells, as well as a reduction of antigen specific antibody (4). Besides direct B-T_{FH} contact, T_{FH} cells also contribute to the GC reaction (GCR) through soluble mediators like IL-4 and IL-21 (9, 10). A recent report claimed distinct subpopulations of

blood CXCR5$^+$ T cells can be found in humans producing cytokines characteristic of Th1, Th2, and Th17 responses (11).

Deregulation of proliferation, mutation and differentiation in GCs can lead to detrimental outcomes, including oncogenesis and immunodeficiency (12, 13). Moreover, it has been shown that mutant B cells with self-specificity can be supported in GCs and contribute to autoimmunity (14). Therefore, regulation of the quality and quantity of plasma cells and memory B-cell populations in GCs is very important to prevent immunopathology.

How this regulation is achieved remains poorly understood. A potential mechanism for this could be the presence within the polyclonal follicular T cell population in GCs of some regulatory T cells with specificity for ubiquitous self-antigens. On the other hand, theoretical modeling points to processes related to T-cell proliferation as being the dominant steps in the GC dynamics (15). All this led us to propose the hypothesis that regulatory mechanisms do exist affecting antigen-specific T_{FH} cells in order to prevent too intense GCRs and/or production of autoantibodies. A possible basis for some of the required GC regulation would be the involvement of Foxp3$^+$ regulatory T (Treg) cells during the GCR.

Currently, little is known about Treg cells in connection with GCs. Some Treg cells are known to express high levels of CXCR5 and to display positive chemotaxis toward a CXCL13 gradient *in vitro* (16). Furthermore, CD25$^+$ T cells (presumably Treg cells) were already shown to be present in the GCs of human tonsils and to suppress *in vitro* T cell activation and immunoglobulin production by B cells (16). In contrast, it was shown that within peyer patches, Treg cells can originate T_{FH} cells (17).

In this report, we show that in murine lymph nodes Treg cells do participate in the GCR, contributing to both the dynamics and the amplitude of the GCR. Additionally, we

show that those follicular Treg cells have an impact on antibody production during a T-dependent immune response.

Materials and Methods

Mice and immunization. Balb/c, C57Bl/6 CXCR5$^{-/-}$, OT2.Rag2$^{-/-}$ mice, Foxp3^{gfp} knock-in mice (generously provided by A.Y. Rudensky), and TCRα$^{-/-}$ mice (from S. Tonegawa) were maintained in SPF facilities). Procedures were conducted in accordance with guidelines from the Animal User and Institutional Ethical Committees.

Unless otherwise stated in the text, animals were immunized i.p. with 20 µg of OVA (Sigma, St Louis, USA) previously run through a DetoxyGel column (Pierce, Rockford, USA) in 2.0 mg of endotoxin-free aluminum hydroxide (alum, Alu-gel-S, Serva, Heidelberg, Germany).

Adoptive transfer. For adoptive cell transfers single cell suspensions from a pool of spleen and LN were sorted based on expression of CD4, CXCR5, PD-1, CD25, and GFP (cells from Foxp3^{gfp} reporter mice) in a FACS Aria (BD, Franklin Lakes, USA), with doublet exclusion in all experiments. Unless otherwise stated in the text 1x10^4 cells of the indicated cell population were injected intravenously into TCRα$^{-/-}$ mice. In all transfer experiments mice were immunized with OVA-alum one day after adoptive transfer.

Flow cytometry. Single cell suspensions of dLNs were analyzed by flow cytometry using mAb targeting: PD-1 (J43), CD4 (L3T4), Thy1.2 (53-2.1), Foxp3 (FJK-16s), Bcl6 (GI191E), GITR (DTA-1), CD103 (2E7), CD69 (H1.2F3), CD25 (all from eBiosciences); and anti-CXCR5 (2G8) and anti-Rat IgG2a (R35-95) (BD Bioscience, San Diego, USA). Foxp3 staining was performed using the Foxp3 staining Set (ebioscience) following the manufacturer's instructions.

In vitro conversion. Sorted populations of $5x10^4$ CD4 T cells were incubated 3 days with 3 µg/ml plate-bound anti-CD3 (145-2C11, eBiosciences), 2 µg/ml soluble anti-CD28 (eBiosciences), and 5 ng/ml TGFβ (R&D Systems).

Suppression assay. Sorted populations of $Foxp3^{gfp+}$ T cells were co-cultured in Terasaki plates (Greiner, Frickenhausen, Germany) with gamma-irradiated APCs, and $Foxp3^{gfp-}$ effector cells (1:3:1 ratio). Cultures were supplemented with 2.5 µg/ml soluble anti-CD3 for 3 days, with addition of 1 µCi ^{3}H-thymidine (Amersham, Sunnyvale, CA) in the last 12h.

Confocal microscopy. 20 µm cryosections fixed in acetone (Sigma) and 50 µm vibratome sections from PFA (Sigma) fixed tissue were obtained from dLN. For Foxp3 staining sections were permeabilized with the Fix/Perm buffer from the Foxp3 staining Set (eBioscience). Samples were stained with the following primary antibodies: rabbit anti-CD3, anti-Ki67, anti-GFP, anti-OVA; and rat anti-CD3 (Abcam), anti-IgM-TxRd (SouthernBiotech, Birmingham, USA), anti-KJ-FITC (Caltag, Carlsbad, USA), anti-CD4-alexa647 (Serotech), PNA-FITC and PNA-bio (Vector, Burlingam, USA). As secondary antibodies we used: anti-FITC-alexa488, anti-rabbit Ig-alexa488, anti-rabbit Ig-alexa647, anti-rat Ig-alexa633 (Invitrogen, Carlsbad, USA); and avidin-rhodamin (Vector) and streptavidin-DyLight488 from ThermoScientific (Massachusetts, USA). Images were acquired using a LSM710 confocal microscope (Zeiss, Jena, Germany) equipped with a 5x (0,16 NA, Zeiss), 10x (0,30 NA, Zeiss), 20x (0,80 NA, Zeiss) and a 40x (1,30 NA, Zeiss) objective. Image analysis was performed using LSM Image Browser.

ELISA assay. Isotype-specific antibody titers in the serum were determined by ELISA on 96-well plates (BD Falcon) coated with 50 µg OVA (grade V, Sigma) in carbonate buffer pH=9.6 + N_3Na 0.01%. Nonspecific binding was blocked with 1% BSA (AMRESCO, Inc.) in PBST. Sera were diluted 1:50 or 1:100 in PBST, and threefold

serial dilutions of serum samples were incubated. Negative controls (normal mouse serum diluted 1:100) were included in all assays. The plates were washed and 50 µl isotype-specific goat anti-mouse-horseradish peroxidase conjugates (SouthernBiotech) was added. ELISAs were developed with ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate (Sigma-Aldrich). The optical density (OD) at 405 nm was determined after 4, 5, and 6 min with an automatic ELISA plate reader (Envision Multilabel Reader 2104, Perkin Elmer) and the highest values were recorded.

Statistical analysis. Statistical significance was determined using the two-tailed non-parametric Mann-Whitney test and P values <0.05 were deemed significant (*, P<0.05; **, P<0.01; ***, P<0.001).

Results

Co-development of the GCR and GC T cells

To analyze GC T cells, Balb/c mice were immunized with Ovalbumin (OVA) to trigger a GCR and sacrificed at days 0, 4, 12 and 16 in order to evaluate the initiation, peak and decline of GCR. Draining lymph nodes (dLN) were collected and 20 µm sections were stained with PNA and anti-CD3 to identify GCs and T cells. After immunization the GCR peaked at about day 12 or a few days before as measured by the average GC volume using confocal microscopy (Figure 1A,B).

The quantification of total T cells within the GCs revealed that the kinetics of T cell numbers parallels that of the global GCR (Figure 1C). However, since GC volume increases during GCR, we calculated the density of T cells within the GC (i.e. number of T cells per unit of GC volume). We found that the T cell density was highest at day 4 post-immunization (Figure 1D), although the real peak is probably a few days later.

Follicular T cells contain a Foxp3$^+$ subset of suppressor cells

Given the observed changes in the density of T cells within the GC during a GCR, we investigated the putative presence of an active regulatory mechanism. Although Treg cells were previously observed in germinal centers (16, 18), the precise nature of follicular Treg cells, their relationship with extrafollicular counterparts, and their *in vivo* functional significance has remained unknown. We therefore investigated whether follicular Treg cells represent a significant subpopulation of the T cells found in the GC.

As illustrated in Figure 2A, Foxp3$^+$ cells were readily detectable in GCs of dLNs. Interestingly, we found that the density of Foxp3$^+$ T cells peaked at day 12 post-immunization (Figure 2B), at the time when the density of T cells was reduced (Figure 1D) and the contraction phase of the GCs was initiated. We confirmed these data using Foxp3^{gfp} knock-in mice (where Foxp3 and GFP are fused together), showing a perfect overlap of Foxp3 staining and GFP expression (Supplementary Figure 1A). By analyzing non-consecutive sections from the same LN, we also established the reliability of the quantitative approach we used (Supplementary Figure 1B).

To further characterize the GC Foxp3$^+$ cells that we observed by confocal microscopy, we analyzed follicular T cells of dLN by flow cytometry. We confirmed the existence of a Foxp3$^+$ subpopulation within follicular T cells (Figure 2C). We found that follicular Foxp3$^+$ T cells share several markers with “conventional” Treg cells, such as high levels of CD25, GITR, or CD103, but also retain the expression of molecules characteristic of T_{FH} cells, such as CXCR5 and PD-1, and the lineage-specific transcription factor Bcl-6 (Figure 2C). Furthermore, we found that FACS-sorted follicular Foxp3$^+$ T cells (sorted based on CD4, PD-1, CXCR5 and Foxp3^{gfp} expression) had similar immune suppressive potential as non-follicular Foxp3$^+$ T cells (Figure 2D).

Therefore, Foxp3$^+$ T cells found in GCs and B cell follicles appear to represent a subpopulation of follicular T cells, bearing a distinctive phenotype from the extrafollicular conventional Treg cells. Importantly, Bcl-6 was recently identified as the master transcription factor for the T_{FH} lineage, by turning on a wide-spread gene repressor program acting on key transcriptional regulators of other T helper cell lineages, namely Tbet (Th1) and RORγt (Th17), as well as a large number of miRNAs (4, 5, 19). It is important to note, however, that a repressive function towards the Treg lineage, or its key regulator Foxp3, was not found.

The increase density of Foxp3$^+$ GC T cells correlates with their increased proliferation as the GCR develops

We next assessed the proliferative behavior of GC Foxp3$^+$ T cells by quantifying the number of these cells positive for the proliferation marker Ki67. As shown in Figure 3, the frequency of proliferating (Ki67$^+$) Foxp3$^+$ cells within the GC is greater than the frequency of proliferating Foxp3$^+$ cells outside the GC. In addition, there is a significant increase of proliferation at day 12 post-immunization – a time that, as discussed above, correlates with an increase in the frequency of Foxp3$^+$ cells within the GC. It is noteworthy that at day 4 post-immunization the proliferation of Foxp3$^+$ cells increases significantly outside the GC. On the contrary, the frequency of proliferating cells within total GC T cells does not change during the GCR.

Foxp3$^+$ GC T cells regulate the magnitude of GCR

After observing an increased frequency of Foxp3$^+$ GC T cells at day 12 post immunization, we assessed the functional significance of such cells for the regulation of GCR. For this purpose we used TCRα$^{-/-}$ mice, which lack α/β T cells and hence also Treg cells, and reconstituted those mice with OVA-specific CD4 T cells from OT2.Rag2$^{-/-}$ mice (that also lack Foxp3$^+$ T cells). The OT2 cells were transferred alone, or together

with sorted Treg cells. We took advantage of Foxp3^{gfp} knock-in reporter mice to isolate by flow cytometry a population of Foxp3^{gfp+} Treg cells. The recipient mice were immunized with OVA, and GCR was followed at different time points. We studied GC resolution at day 20, instead of day 16, in order to have better discrimination concerning a putative impact on the serum titre of switched immunoglobulins.

Since TCRα$^{-/-}$ mice are known to show an impaired ability to develop GCs due to their lack of α/β T cells (20), we first wanted to ensure that TCRα$^{-/-}$ mice could develop GCs after adoptive cell transfer of antigen-specific cells. Lymph node sections from TCRα$^{-/-}$ mice transferred with OTII cells and immunized with OVA-alum were analyzed for the presence of GCs by staining with PNA and anti-IgM. This study revealed the ability of OT2 cells to mount a GCR (Figure 4A), with a kinetics having a relatively high magnitude (Figure 4B). However, co-transfer of Foxp3^{+} Treg cells together with OT2 cells, reduced significantly the magnitude of the GCR (Figure 4B). Consistent with this finding, we also found that the adoptive transfer of Foxp3^{+} Treg cells had an impact on antibody production, leading to lower serum titers of secreted OVA-specific IgG1, IgM, IgG2b and IgA (Figure 4C). The Ab titres were reduced approximately one log by day 20, but with mice transferred with Treg cells still showing lower serum titres (Figure 4C,D).

However, given the known ability of Foxp3^{+} Treg cells to suppress T cells responses, it was possible that the observed regulation was acting on the generation of helper T cells during their early stage of activation taking place in the T zone of the LN, and not within the GC. To address this issue we took advantage of CXCR5$^{-/-}$ mice. These mice provided the tool to discriminate between the *in vivo* physiological impact of Treg cells within and outside the follicle, as Treg cells from CXCR5$^{-/-}$ mice have a similar function to wild type Treg cells but cannot enter the follicle. Therefore, we repeated the experiment performing an adoptive transfer of OT2 cells into TCRα$^{-/-}$ mice. Together

with the OT2 cells we transferred flow cytometry sorted $CD4^{+}CD25^{bright}$ T cells (that we confirmed were predominantly $Foxp3^{+}$) from $Foxp3^{gfp}$ or $CXCR5^{-/-}$ mice. As anticipated, Treg cells from $CXCR5^{-/-}$ mice could not be found within GC, while the effector OT2 cells could still be found in the GC (Figure 4E-G). Moreover, the ratio of effector/regulatory cells observed in the GC of $TCR\alpha^{-/-}$ mice transferred with T cells was similar to wild type conditions (Figure 4E, F), although with a smaller number of cells. The ratio of follicular Treg/Teffector cells is not too different from the ratio observed within conventional extra-follicular populations. Remarkably, we were able to show the physiological role of follicular $Foxp3^{+}$ T cells, as we found that adoptively transferred $CXCR5^{-/-}$ Treg cells could not reproduce the phenotype observed with wild-type Treg cells (Figure 4H).

Taken these results together, it appears that CXCR5 expression by Treg cells is essential to endow them with the ability to control the GCR. We propose, therefore, naming this $Foxp3^{+}$ T cell subset from GCs that express PD-1, CXCR5, and Bcl-6, as regulatory follicular T cells ($T_F$reg).

Follicular $Foxp3^{+}$ T cells derive from conventional Treg cells

We then investigated whether $Foxp3^{+}$ follicular T cells derive from conventional T cells or from natural Treg cells that acquire a follicular phenotype. We confirmed, as anticipated, that the thymus does not contain a population of $Foxp3^{+}$ T cells with follicular characteristics (Figure 5A), indicating the follicular $Foxp3^{+}$ T cells acquire their phenotype in the periphery.

In order to establish the origin of follicular $Foxp3^{+}$ cells, we adoptively transferred sorted T_{FH} or conventional $CD25^{-}$ CD4 T cells from wild-type mice, together with $PD\text{-}1^{-}$ $CXCR5^{-}Foxp3^{gfp+}$ natural Treg cells into $TCR\alpha^{-/-}$ mice, that was subsequently immunized with OVA-alum. Since, adoptive transfer of a CD4 population devoid of Treg

cells does lead to inflammatory bowel disease (21), control mice that did not receive Treg cells were transferred with OT2 cells, as these TCR-transgenic T cells do not lead to autoimmunity in the absence of Tregs. We found in both cases that the majority of follicular Foxp3^{+} T cells recovered from the TCRα$^{-/-}$ mice had derived from the transferred Treg population, as GFP is expressed together with Foxp3 (Figure 5B). Intriguingly, that fraction was larger when T_{FH} cells were transferred together with Foxp3^{gfp+} Treg cells, suggesting a role for T_{FH} cells in recruiting Foxp3^{+} follicular T cells, perhaps by promoting their proliferation. This issue remains to be further investigated.

Furthermore, we sorted T_{FH} and conventional CD4 T cells, that were stimulated *in vitro* with plate-bound anti-CD3 in presence of anti-CD28 and TGFβ – conditions that are known to induce Foxp3 expression (22)(23). We found that T_{FH} cells are resistant to conversion towards a Foxp3^{+} phenotype (Figure 5C).

Taken together, these data suggest that $T_F$reg cells derive predominantly from natural Treg cells, while T_{FH} cells are not amenable to TGFβ-dependent conversion into Foxp3^{+} regulatory cells.

Discussion

Due to the absence of experimental data, there are various mathematical models trying to explain the regulation and termination of the GCR. One hypothesis, is an increasing impairment of the engagement of B cell receptors and antigen through either antigen consumption (24) or antigen masking (25). Both of these concepts assume that GC termination is regulated by the amount of antigen presented within the GC by follicular dendritic cells (FDC). Another hypothesis suggests GC termination is caused by increased differentiation of GC B cells towards memory and plasma cells due to signals from FDCs (15). A third hypothesis proposes that signaling from T cells is the responsible for GC termination (15). After observing an increase in $T_F$reg cell numbers

in the GC towards the end of the GCR, we suggest a wider scenario whereby this cell population plays a role in controlling the magnitude and duration of the GCR.

The lack of Treg cells in our transfer system profoundly modified the outcome of the GCR, as those mice showed larger GCs at day 12, and greater production of different classes of Ig, than in the presence of Treg cells. Moreover, using Treg cells isolated from CXCR5$^{-/-}$ mice, we created a system that has Treg cells but those are unable to gain access to GC follicles. Thus the data showed that it is specifically the $T_F$reg cells that control the GCR. Although our data do not prove unequivocally a role for $T_F$reg cells in the termination of the GCR, they clearly show that $T_F$reg cells affect the magnitude of this reaction from mid-term on. To date it is not clear whether this regulation operates by controlling effector T_{FH} cells (16), and by that indirectly affecting GC B cells, or if that regulation acts directly on GC B cells or on both T_{FH} and B cells (18).

A second outstanding question concerns the origin of $T_F$reg cells. We could not detect Foxp3$^+$ cells with a T_{FH}-like phenotype in the thymus. Furthermore, when we transferred OT2 cells into TCRα$^{-/-}$ mice we could not find *in vivo* conversion of those Foxp3$^-$ cells into $T_F$reg cells. But when we transferred natural Treg cells together with CD4 T cells, some of the natural Treg cells (the only ones expressing Foxp3^{gfp} in those mice) acquired access to the follicles and phenotypic follicular characteristics. Therefore, we believe it is likely that in the same way that natural Treg cells can originate T_{FH} cells in the PP (17), some natural Tregs can also differentiate towards a $T_F$reg phenotype in LNs and the spleen. However, this does not seem to be the only origin of $T_F$reg cells since 25% to 50% of $T_F$reg cells originated from transferred CD25$^-$ CD4$^+$ T cells. This has important implications for understanding how the dynamics of T and B cells within GCs is controlled.

GC deregulation or malfunctions are linked to the onset of severe diseases, as cancer and autoimmunity (26). Taken together, our data show that follicular T cells include a new regulatory subpopulation, which by controlling the GCR may ultimately impact on preventing such pathologies.

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Authorship

Contribution: I.W., A.A.D., A.H. C.A. and V.G.O. performed experiments; I.W., A.A.D., A.H., C.A., J.F. and L.G. analyzed results; I.W. made the figures; I.W., J.F. and L.G. designed the research and wrote the paper.

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Footnotes

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Abbreviations used: GC, germinal center; GCR, germinal center reaction; OVA, Ovalbumin; PD-1, programmed death 1; Tfh, follicular helper T cell; Tfreg, follicular regulatory T cell; Treg, regulatory T cell.

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Figure legends

Figure 1. *Kinetics of GCR and GC T cells.* Balb/c mice were immunized with 20 μg OVA-alum i.p. and sacrificed at the indicated time points, when cryosections of mesenteric lymph nodes were analyzed to follow the GCR. (A) Average GC volume. GCs were identified by PNA staining and quantified by confocal microscopy. (B) Representative stainings from non-immunized mice, and mice sacrificed on days 4, 12 and 16 post immunization. Bar: 500 μm. (C) Total number of T cells, and (D) density of GC T cells during a GCR as analyzed by confocal microscopy. GC T cells were identified as $CD3^+$ cells within the PNA area. (E) Representative staining from a LN on day 12 after immunization. Bar: 50 μm. Data are representative of two independent experiments. *** $P<0.001$ (Mann-Whitney non-parametric, two-tailed test).

Figure 2. *$Foxp3^+$ T cells can be found within the GC and represent a subpopulation of follicular T cells.* Balb/c mice were immunized with 20 μg OVA-alum i.p. and sacrificed

at the indicated time points. (A) Cryosections of dLN were stained with PNA and anti-Foxp3 to identify $Foxp3^+$ cells within the GC. Bar: 20 µm. (B)The density of $Foxp3^+$ T cells within the GC during the course of a GCR was quantified by confocal microscopy. The density corresponds to the total number of $Foxp3^+$ cells within the PNA^+ area divided by the measured GC volume. (C) $CD4^+$ lymphocytes from dLN of mice immunized with OVA-alum 12 days before were studied by flow cytometry. T_{FH} cells were defined as being $CXCR5^+PD1^+$ as defined by the represented gate. The conventional extra-follicular T cells (non-T_{FH}) were defined as the $CXCR5^-PD\text{-}1^-$ cells. T_{FH} cells had higher expression of Bcl-6 (top right). When we studied the T cells within the T_{FH} gate (middle row) and non-T_{FH} gate (bottom row), we found both populations had a proportion of cells expressing Foxp3. We compared the expression of the indicated molecules within the $Foxp3^+$ and $Foxp3^+$ populations of T_{FH} and non-T_{FH} cells. (D) Follicular $Foxp3^+$ T cells ($CD4^+CXCR5^+PD1^+Foxp3^+$) and non-follicular conventional Treg cells ($CD4^+CXCR5^-PD\text{-}1^-Foxp3^+$) were sorted from the spleen and LNs of $Foxp3^{gfp}$ reporter mice. $2x10^3$ Treg cells were co-cultured in triplicate with gamma-irradiated APCs and $Foxp3^{gfp-}$ effector cells (1:3:1 ratio). Cultures were supplemented with 2.5 µg/ml soluble anti-CD3 Ab for 3 days and ^3H-thymidine was added in the last 12 hours. Data are representative of two independent experiments. * $P<0.05$, ** $P<0.01$ (Mann-Whitney non-parametric, two-tailed test).

Figure 3. *Kinetics of proliferation of T cells and $Foxp3^+$ T cells during the GCR.* Balb/c mice were immunized with OVA-alum as described above. The frequency of proliferating T cells was quantified by confocal microscopy based on Ki67 expression. (A) Proliferating GC $Foxp3^+$ cells and GC T cells were identified as, respectively, $Ki67^+Foxp3^+$ cells and $Ki67^+CD3^+$ cells within the PNA area, and are depicted as % of the total $Foxp3^+$ or total $CD3^+$ cells identified in the GC. (B) A similar analysis was performed for T cells outside the GC. (C) Representative stainings of proliferation of

GC T cells. Bar: 50 μm. Data are representative of two independent experiments. * $P<0.05$, ** $P<0.01$ (Mann-Whitney non-parametric, two-tailed test).

Figure 4. *Follicular Foxp3$^+$ T cells regulate the magnitude of GCR in vivo.* (A) TCRα$^{-/-}$ mice were adoptively transferred with 10^4 OVA-specific T cells from OT2.Rag2$^{-/-}$ mice. Some mice also received an equal number of Foxp3^{gfp+} T cells. All mice were immunized with OVA-alum. The micrographs show GC in mice that did not receive Foxp3$^+$ T cells at day 12 post-immunization. Top bar: 200 μm, bottom bar: 50 μm. (B) Quantification of the GC volume from dLN immunized with OVA-alum in the presence or absence of adoptively transferred Foxp3$^+$ T cells. GCs were identified as a PNA$^+$ region within the follicle marked by IgM. (C,D) Serum titres of OVA-specific immunoglobulins of different classes were determined by ELISA at different times after immunization. In the absence of Treg cells the mice produced significantly higher levels of IgM, IgG1, IgG2b, and IgA. (E) Quantification of total CD4$^+$ T cells and (F) Foxp3$^+$ Treg cells within GCs, showing that following adoptive cell transfers of defined T cell populations into T cell-deficient mice there was a reduced number of both T and Treg cells compared with wild-type mice, although their ratio was roughly maintained. (n.d. – not detected). (G) Representative micrographs showing that it was possible to identify Foxp3^{gfp+} T cells within day 12 GCs of mice adoptively transferred with Treg cells, but not in mice transferred with CXCR5$^{-/-}$ Treg cells, where Treg cells remained outside the follicle. Bar: 20 μm. (H) TCRα$^{-/-}$ mice were adoptively transferred with Treg cells sorted from wild-type or CXCR5$^{-/-}$ mice, and immunized with OVA-alum as described in (A). The GC volume was quantified as detailed above. Data are representative of two independent experiments. * $P<0.05$, ** $P<0.01$, * $P<0.001$ (Mann-Whitney non-parametric, two-tailed test).

Figure 5. *Follicular Foxp3$^+$ T cells originate from conventional Treg cells.* (A) Among CD4$^+$Foxp3$^+$ thymocytes, we could not find cells expressing CXCR5 and PD1. (B)

TCRα$^{-/-}$ mice were adoptively transferred with equal numbers of PD-1^{-} CXCR5^{-} Foxp3^{+} Treg cells sorted from Foxp3^{gfp} knock-in mice, and CD4^{+}CD25^{-} T cells (T_{FH}: PD-1^{+}CXCR5^{+} or CD4: PD-1^{-}CXCR5^{-}) from B6 mice. Control mice were transferred with Foxp3^{-} OT2 cells alone. All mice were immunized with OVA-alum. The dot plots represent follicular CD4^{+} T cells, gated in the PD-1^{+}CXCR5^{+} region, analyzed for Foxp3 and GFP expression. The majority of Foxp3^{+} cells are also GFP^{+}, indicating they derived from adoptively transferred Treg cells (from Foxp3^{gfp} mice). Representative data from 4 mice per group. (C) Foxp3^{gfp-} T_{FH} (sorted as CD4^{+}PD-1^{+}CXCR5^{+}) and non-T_{FH} cells (sorted as CD4^{+}PD-1^{-}CXCR5^{-}) were cultured for 3 days with plate-bound anti-CD3 in presence or absence of 5 ng/mL TGFβ. The frequency of cells converted into Foxp3^{+} was analyzed at the end of the culture (n=3, representative of two independent experiments).

Supplemental Figure 1. *Validation of the quantitative studies.* (A) Cryosections from LNs of Foxp3^{gfp} knock-in mice, previously immunized with OVA-alum, were stained with anti-Foxp3 and anti-GFP antibody to confirm that both molecules allowed the identification of the same cells. Bar: 50 μm. (B) Balb/c mice were immunized with OVA-alum i.p. and non-consecutive cryosections from the same LN were studied at different time points. The quantified cells were identified as the Foxp3^{+} cells within the PNA area.

Figure1

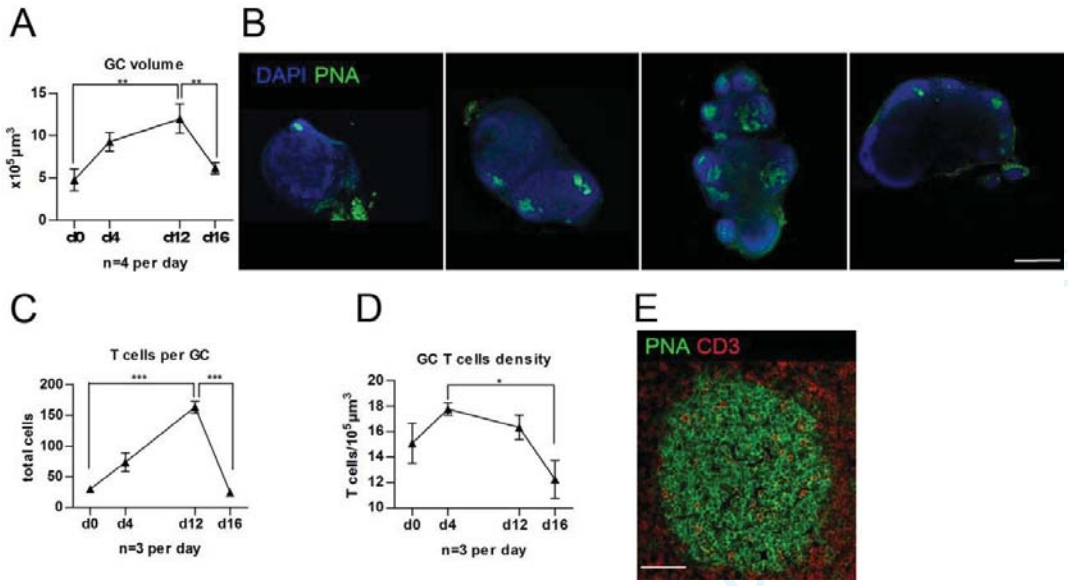


Figure 2

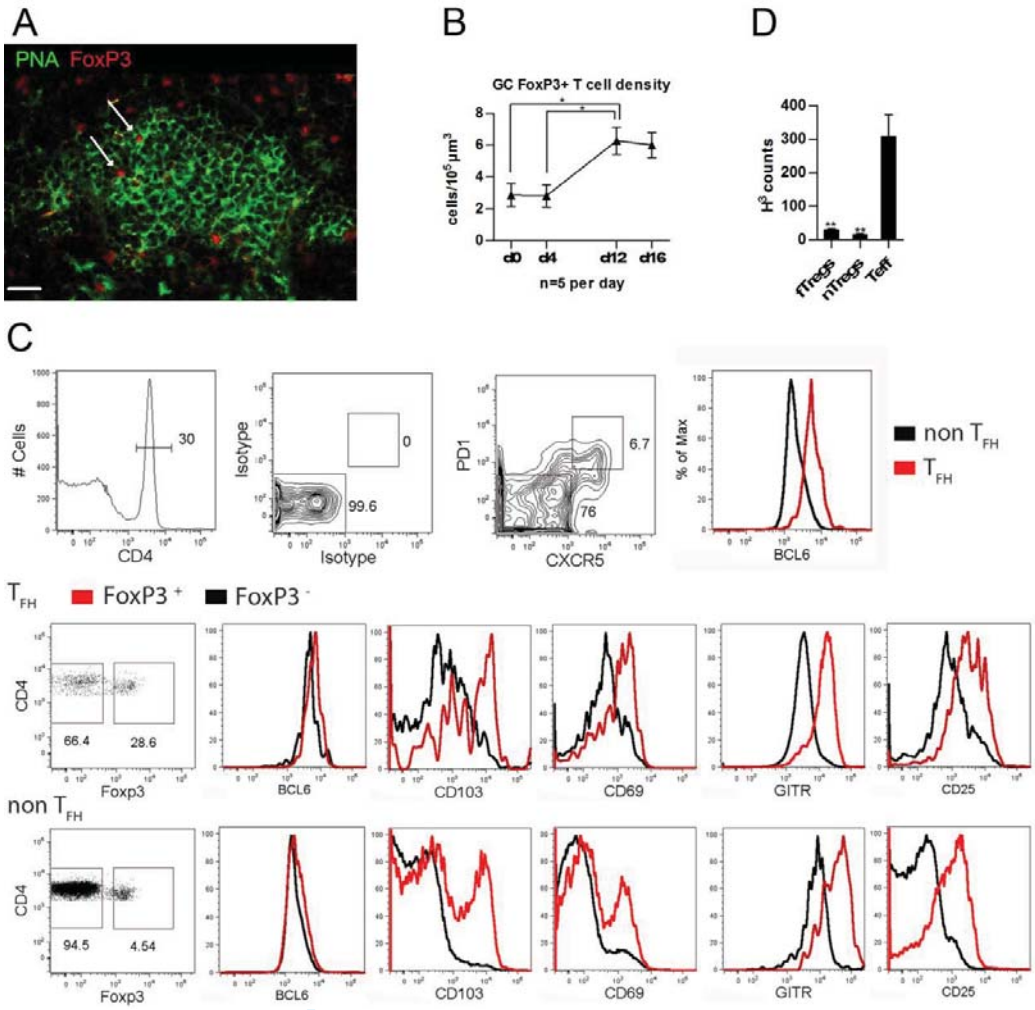


Figure 3

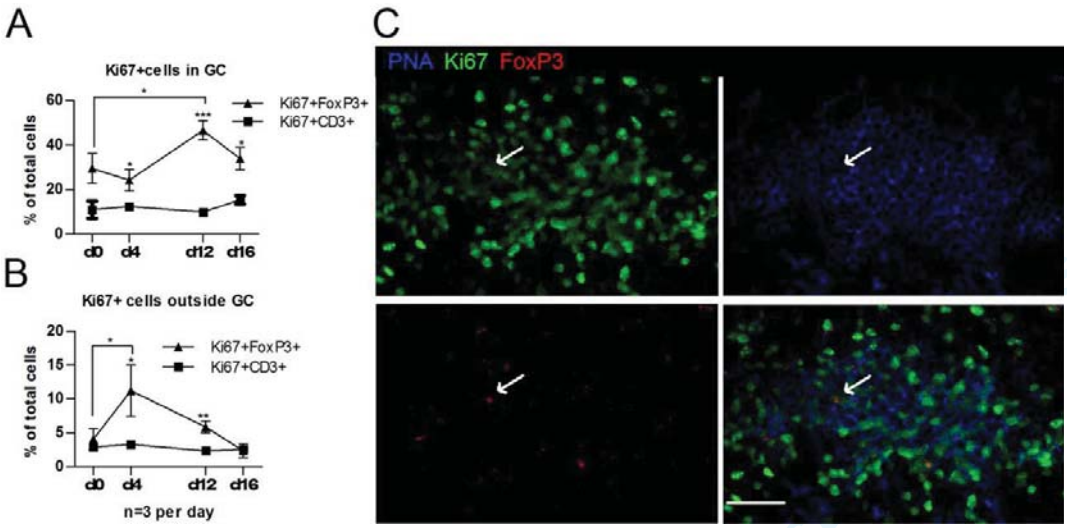
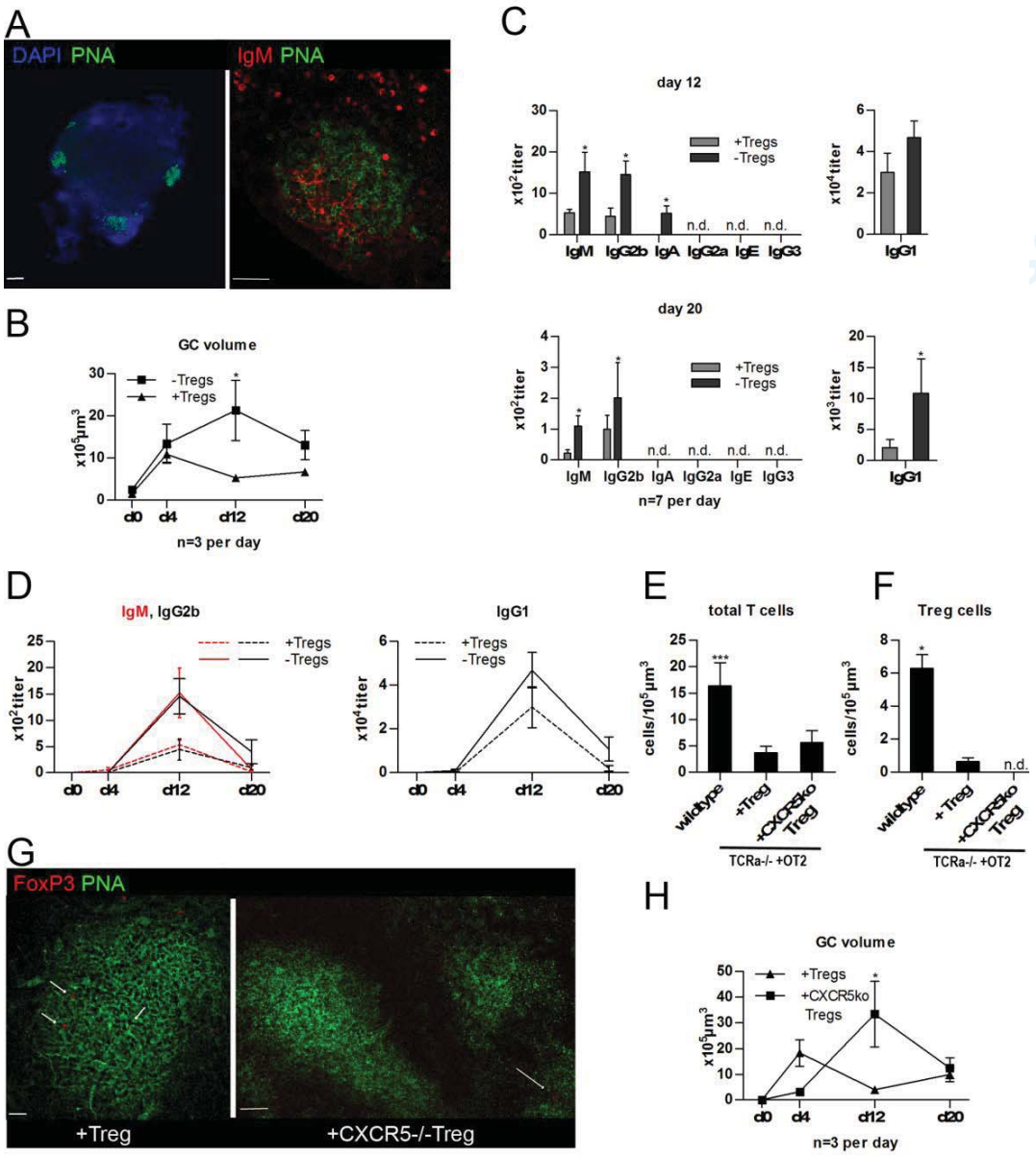


Figure 4


A
DAPI PNA
IgM PNA
B
GC volume
-Tregs
+Tregs
x10^5µm^3
d0 d4 d12 d20
n=3 per day
C
day 12
+Tregs
-Tregs
x10^2titer
IgM IgG2b IgA IgG2a IgE IgG3
n.d.
x10^4titer
IgG1
day 20
x10^3titer
n=7 per day
D
IgM, IgG2b
IgG1
E
total T cells
cells/10^5µm^3
wildtype
+Treg
+CXCR5ko Treg
TCRa-/- +OT2
F
Treg cells
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FoxP3 PNA
+Treg
+CXCR5-/-Treg
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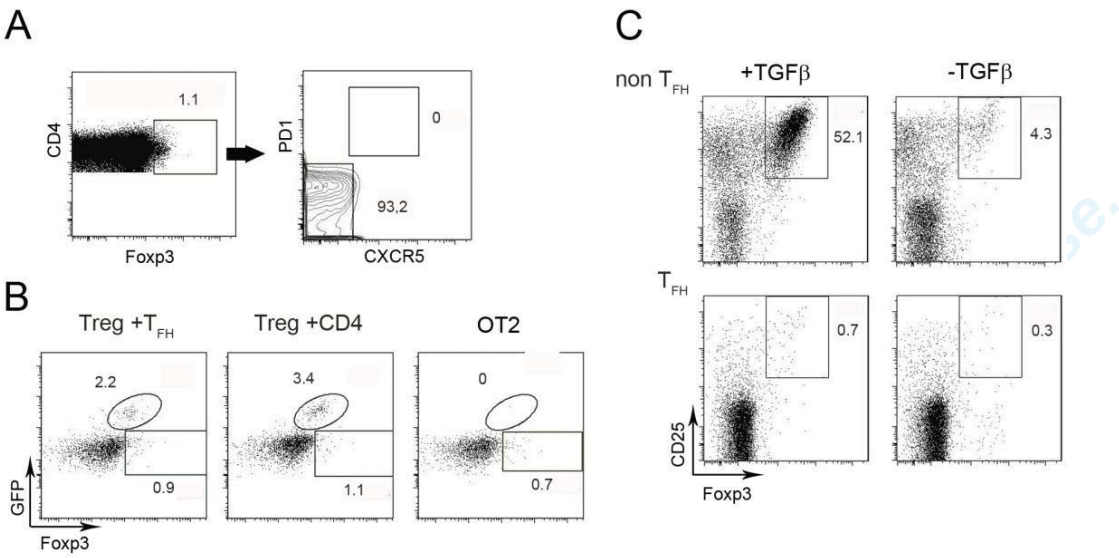
+CXCR5ko Tregs

Figure 5



Identification of Regulatory Foxp3$^+$ Invariant NKT Cells Induced by TGF- β

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Invariant NKT (iNKT) cells were shown to prevent the onset of experimental autoimmune encephalomyelitis in mice following administration of their specific TCR agonist α-galactosylceramide. We found that this protection was associated with the emergence of a Foxp3$^+$ iNKT cell population in cervical lymph nodes. We demonstrate that the differentiation of these cells is critically dependent on TGF-β in both mice and humans. Moreover, in vivo generation of Foxp3$^+$ iNKT cells was observed in the TGF-β–rich environment of the murine gut. Foxp3$^+$ iNKT cells displayed a phenotype similar to that of Foxp3$^+$ regulatory T cells, and they suppress through a contact-dependent, glucocorticoid-induced TNFR-mediated mechanism. Nevertheless, Foxp3$^+$ iNKT cells retain distinctive NKT cell characteristics, such as promyelocytic leukemia zinc finger protein expression and preferential homing to the liver following adoptive transfer, where they stably maintained Foxp3 expression. Our data thus unveil an unexpected capacity of iNKT cells to acquire regulatory functions that may contribute to the establishment of immunological tolerance. ***The Journal of Immunology*****, 2010, 185: 2157–2163.**

Natural killer T cells are innate-like lymphocytes capable of producing cytokines characteristic of Th1, Th2, or Th17 responses (1–3). They were shown to influence adaptive immunity by exacerbating or suppressing a diversity of immune disorders, such as allergy, autoimmunity, or transplantation (4–7). For instance, NKT cell activation following administration of α-galactosylceramide (α-GalCer), an NKT cell-specific agonist, can protect mice from experimental autoimmune encephalomyelitis (EAE) manifestations (5, 8).

NKT cells express a TCR and receptors typical of the NK lineage, including NK1.1 and NKG2D (7). The best-studied NKT cell subset, known as type I, classical, or invariant NKT (iNKT) cells, has a semi-invariant TCR comprising an invariant α-chain and a restricted TCR β-chain repertoire. These cells recognize glycolipids presented by CD1d (7) and develop in the thymus, where they undergo a positive selection process mediated by double positive thymocytes acting as CD1d$^+$ APCs, instead of classical MHC-expressing thymic epithelial cells (9). Thus, iNKT cells are generally considered a lineage separate from T lymphocytes, characterized molecularly by expression of promyelocytic leukemia zinc finger protein (PLZF) (10). Of note, their unique TCR remains the only distinctive feature enabling iNKT cell unambiguous detection, because they share many surface molecules with T cells, namely, CD3 and CD4 in mice, as well as CD8 in humans (7, 11).

Thus far, iNKT cell subsets identified in vivo can recapitulate in the innate immunity context some of the functions characteristic of conventional CD4 T lymphocytes, such as secretion of Th1, Th2, and Th17-type cytokines (1–3, 12, 13). However, a parallel between regulatory CD4 T cells (regulatory T [Treg] cells) and iNKT lymphocytes has not yet been described. Treg cells are characterized by expression of the transcription factor Foxp3 and potent immunosuppressive properties. They are crucial for the maintenance of an immunological self-tolerance state, modulating the activation, proliferation, and function of effector T cells, thereby preventing pathological immune responses, including allergy and autoimmunity (14). Treg cells develop in the thymus or in particular contexts at the periphery, when activated in the presence of TGF-β (15–18).

We have now identified a population of Foxp3$^+$ iNKT cells in cervical lymph nodes (CLNs) of mice protected from EAE following α-GalCer administration. We further demonstrate that murine and human Foxp3$^+$ iNKT cells can be induced in vitro following activation in the presence of TGF-β. Foxp3$^+$ iNKT cells display Treg cell phenotypic hallmarks, including CD25, glucocorticoid-induced TNFR (GITR), and CTLA-4, while retaining NKT cell characteristics, namely, PLZF expression. Moreover, Foxp3$^+$ iNKT cells can occur in vivo, following activation in the TGF-β–rich mucosal environment. Because of the strong suppressive properties displayed upon Foxp3 upregulation, we termed this new NKT cell subset "Foxp3$^+$ NKTreg cell".

Materials and Methods

Mice

C57BL/6J (H-2^b, B6), B6.Cg-*Igh*^{a}*Thy1*^{a}*Gpi1*^{a}/J (H-2^b, Thy1.1), BALB/cByJ (H-2^d, BALB/c), and B6.Cg-Tg(Cd4-TGFBR2)16Flv/J (H-2^b, dominant negative [dn] TGFβRII) obtained from The Jackson Laboratory (Bar Harbor, ME); B6.129S6-Rag2^{tm1Fwa}N12 (H-2^b, RAG2$^{-/-}$) obtained from Taconic Farms (Germantown, NY); and *FoxP3*^{gfp} knockin mice (H-2^b) obtained from University of Washington (Seattle, WA) were bred and maintained in specific pathogen-free conditions at the Instituto Gulbenkian de Ciência, in Oeiras, Portugal. EAE was induced with 200 μg myelin

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CLN, cervical lymph node; dn, dominant negative; EAE, experimental autoimmune encephalomyelitis; α-GalCer, α-galactosylceramide; GITR, glucocorticoid-induced TNFR; iNKT, invariant NKT; iNKTreg, invariant regulatory NKT; iTreg, induced regulatory T; MLN, mesenteric lymph node; MOG, myelin oligodendrocyte glycoprotein; nTreg, natural regulatory T; pLN, pooled lymph node; PLZF, promyelocytic leukemia zinc finger protein; Treg, regulatory T.

oligodendrocyte glycoprotein (MOG) peptide (Biopolymers Laboratory, Harvard Medical School, Boston, MA) in 200 μg CFA (Difco/BD Diagnostics, Franklin Lakes, NJ) s.c. and 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) i.v. on days 0 and 2. Some mice received 4 μg α-GalCer (Alexis Biochemicals/Enzo Life Sciences, Plymouth Meeting, PA) on days 0 and 4. Disease severity was monitored daily and graded as follows: 1, limp tail; 2, partial hind-leg paralysis; 3, complete hind-leg paralysis; 4, front-leg weakness; and 5, moribund. In gavage experiments, 30 μg α-GalCer was delivered three times every other day.

Human subjects

Peripheral blood samples were obtained from healthy volunteers of both sexes after informed consent. The procedures were reviewed and approved by the Ethical Board of the Faculty of Medicine, University of Lisbon, Lisbon, Portugal.

Flow cytometry and cell sorting

CD1d-PBS57 tetramers were supplied by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). mAbs against mouse CD4, CD25, CD62L, CD103, CD127, CTLA-4, Foxp3, IFN-γ, IL-4, GITR, NK1.1, NKG2D, TCR-β, Thy1.1, Thy1.2; and human CD4, CD25, CD127, CD161, Foxp3, GITR, and TCR Vβ11 were purchased from eBioscience (San Diego, CA), BD Biosciences (San Jose, CA), Beckman Coulter (Fullerton, CA), or BioLegend (San Diego, CA). For murine NKT cell enrichment, cells were incubated with unconjugated anti-CD16/32 Ab (in-house production) to block nonspecific binding to FcR and labeled with PE-conjugated CD1d-PBS57 tetramers without washing. Anti-PE magnetic beads were added, and the magnetically labeled fraction was isolated in an autoMACS Cell Separator (Miltenyi Biotec, Auburn, CA). For human NKT cell enrichment, cells were labeled with biotinylated Abs against CD14, CD19, and CD123, bound to anti-biotin magnetic beads and enriched on an autoMACS Cell Separator, the magnetically labeled fraction being discarded. Intracellular flow cytometry stainings were performed using the Foxp3 Staining Buffer Set (eBioscience) for permeabilization and fixation. Samples were analyzed on a FACSCanto I (BD Biosciences) or sorted on a FACSAria (BD Biosciences), with doublet exclusion in all experiments. Data were analyzed by FlowJo (Tree Star, Ashland, OR).

Cell cultures

Following magnetic enrichment, iNKT cells isolated from mouse spleens were sorted by FACS, and 50,000 cells per well were stimulated with 3 μg/ml plate-bound anti-CD3 (eBioscience). In some experiments, cultures were supplemented with TGF-β (5 ng/ml; R&D Systems, Minneapolis, MN), IL-2 (5 ng/ml; eBiocience), IL-15 (100 ng/ml; eBioscience), and IL-7 (5 ng/ml; R&D Systems). Human cells were stimulated with 1 μg/ml plate-bound anti-CD3 (BD Biosciences). In some conditions, cultures were supplemented with TGF-β (10 ng/ml; R&D Systems), IL-2 (20 U/ml; Roche Diagnostic Systems, Indianapolis, IN), anti–IL-12 and anti–IFN-γ (5 μg/ml; eBioscience), anti–IL-4 (5 μg/ml; R&D Systems), and anti-CD28 (2 μg/ml; eBioscience). Culture medium was RPMI 1640 with GlutaMAX, supplemented with 10% FBS, 1% HEPES, 1% penicillin/streptomycin, 1% sodium pyruvate, and 0.1% 2−ME (Invitrogen, Carlsbad, CA).

In vitro proliferation and suppression assays

Regulatory cells isolated by FACS from the spleen of naive animals (natural Treg [nTreg] cells) or from polarizing cultures (Foxp3$^+$ and Foxp3$^-$ iNKT or CD4 T cells) were cocultured in Terasaki plates (Greiner Bio-One, Frickenhausen, Germany) with mitomycin C (Sigma-Aldrich, St. Louis, MO)-treated splenocytes and responder cells (4000 naive CD4 T cells isolated from spleen; 1:1 ratio with splenocytes) stimulated with 2.5 μg/ml soluble anti-CD3 for 4 d, with addition of 1 μCi [^{3}H]thymidine (Amersham Biosciences/GE Healthcare, Sunnyvale, CA) in the last 12 h. In some experiments, 200 μg/ml anti-IL10R or 100 μg/ml anti-GITR (19) was added. In transwell assays, 100,000 naive CD4 T responder cells labeled with 5 μM CFSE (Invitrogen) were stimulated with mitomycin C-treated splenocytes (250,000) and 1 μg/ml soluble anti-CD3.

Confocal microscopy

Sorted Foxp3-GFP$^+$ cells were plated on coverslips precoated with poly-L-lysine (Sigma-Aldrich) and incubated for 1 h at 37°C. Slides were then incubated with CD1d/PBS57-PE tetramer for 1 h at 4°C, washed with ice-cold PBS, and fixed in PBS 3% paraformaldehyde (Sigma-Aldrich) for 15 min at 4°C. Slides were mounted in DAPI Fluoromount G (Southern Biotech, Birmingham, AL) and analyzed with a laser scanning confocal microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany).

RNA extraction, RT, and PCR

RNA was extracted from 1,000–50,000 cells directly sorted into Buffer RLT with RNeasy Micro Kit (Qiagen, Valencia, CA), and cDNA synthesis was performed using random primers (Invitrogen) and Superscript III RT (Invitrogen). Primers (Bonsai Technologies, Orissa, India) were as follows: PLZF fwd: 5′-cagtttgcgactgagaatgc-3′, rev: 5′-ttcccacacagcagacagaa-3′; Foxp3 fwd: 5′-cccaggaaagacagcaacctt-3′, rev: 5′-ttctcacaaccaggccacttg-3′; EF1A fwd: 5′-acacgtagattccggcaagt-3′, rev: 5′-aggagccctttcccatctc-3′. PCRs were performed using the Power SYBR Green PCR Master Mix and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). All PCR products were run in agarose gel.

Statistical analysis

The p values were calculated by nonparametric unpaired t test with Welch's correction.

Results

Protection from EAE upon α-GalCer treatment leads to the emergence of Foxp3$^+$ iNKT cells in CLNs

Immunization with α-GalCer had been shown to prevent the onset of EAE in wild-type mice (5, 8) (Fig. 1*A*). Because this protection is known to be iNKT cell dependent (20) (Supplemental Fig. 1), we evaluated the number and phenotype of iNKT cells at the peak of disease. Surprisingly, we found a population of Foxp3$^+$ iNKT cells in CLNs (draining the CNS) of mice protected from EAE, which was absent from other anatomical locations and in CLNs of naive animals (Fig. 1*B*, 1*C*, Supplemental Fig. 2). In contrast to Foxp3$^+$ iNKT cells, the number and frequency of Foxp3$^+$ Treg cells remained unchanged in α-GalCer–treated mice, when compared with animals with EAE or naive controls (Fig. 1*D*).

Foxp3 expression has thus far been noted to be confined to conventional αβ T cells (21–23). Consistent with this, we found no evidence for Foxp3 expression by iNKT cells from liver, spleen, pooled lymph nodes, Peyer's patches, or thymus of naive BALB/c or C57BL/6 mice (Supplemental Fig. 3). This finding strongly suggests that Foxp3 expression is not imprinted during thymic iNKT cell development. Instead, our data indicate that peripheral induction of Foxp3$^+$ iNKT cells can occur under particular pathophysiological settings, such as EAE.

TGF-β induces de novo expression of Foxp3 in iNKT cells

We investigated the capacity of iNKT cells to express Foxp3 when activated in the presence of TGF-β—a condition known to convert conventional CD4 T cells into Foxp3$^+$ "inducible" Treg cells (14, 16). Sorted iNKT and CD4$^+$CD25$^-$ T cells were stimulated with plate-bound anti-CD3 in the presence of IL-2 and TGF-β. After 3 d, Foxp3 expression was detectable in a significant proportion of both iNKT (29.35% ± 11.80) and CD4 (53.21% ± 12.03) T cell cultures (Fig. 2*A*, 2*B*). Similar results were obtained with iNKT cells from mice harboring a Foxp3-GFP knockin allele (*Foxp3^{gfp}* mice) (21) and BALB/c mice (Fig. 2*C*, Supplemental Fig. 4), and with iNKT cells sorted from the thymus, albeit the latter exhibited a lower conversion efficiency (Fig. 2*D*). Foxp3^{gfp} iNKT cells were sorted after conversion and individual cells analyzed by confocal microscopy. As shown in Fig. 2*E*, staining with CD1d tetramer loaded with the PBS57 ligand confirms that these Foxp3-expressing cells bear in their surface the invariant TCR that recognizes glycolipid Ags, a feature exclusively attributed to iNKT cells. Therefore, bona fide iNKT cells were similar to conventional CD4 T cells in their ability to upregulate the Foxp3 transcription factor when stimulated under appropriate conditions. Of note, this property was not shared by other unconventional

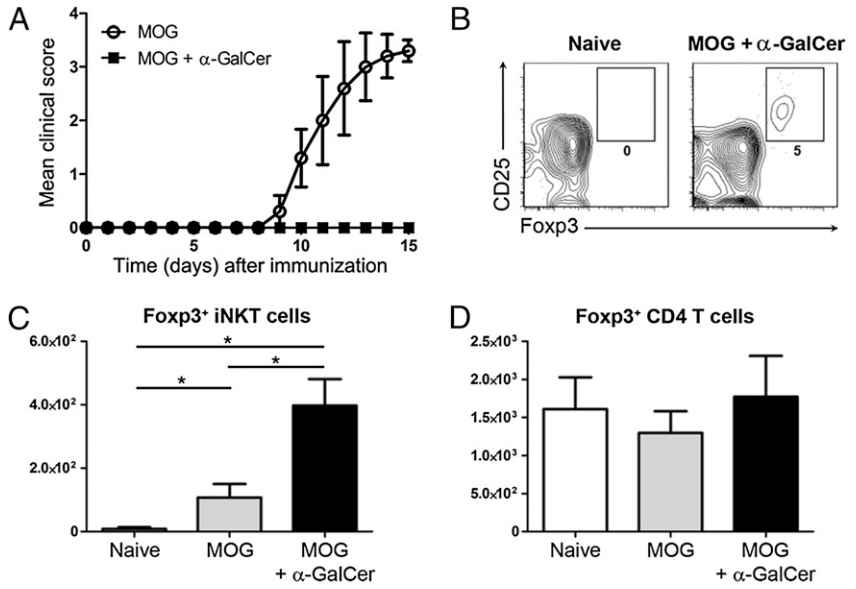

FIGURE 1. Foxp3$^+$ iNKT cells in α-GalCer–treated mice. EAE was induced in C57BL/6 mice (MOG), some of which were treated with α-GalCer (MOG + α-GalCer). *A*, Mice treated with α-GalCer were protected from EAE. $n = 5$; $p < 0.01$. *B*, Representative data from CLNs of naive and α-GalCer–treated mice (gated on iNKT cells, identified as CD1d/PBS57$^+$TCRβ$^+$ cells). *C*, Number of Foxp3$^+$ iNKT cells in CLNs, showing increased Foxp3$^+$ iNKT cell numbers in α-GalCer–treated mice. $n = 5$; $*p < 0.05$. *D*, The number of Foxp3$^+$ Treg cells in CLN remained constant. Data are representative of three independent experiments.

(non–MHC-restricted) T cells, such as γδ T cells, which failed to upregulate Foxp3 upon activation in the presence of TGF-β (M. Monteiro and J. Ribot, unpublished observations).

We analyzed the kinetics of Foxp3 induction and evaluated the stability of Foxp3 expression by iNKT cells in culture for 14 d (Supplemental Fig. 5). We observed that, despite a lower conver-

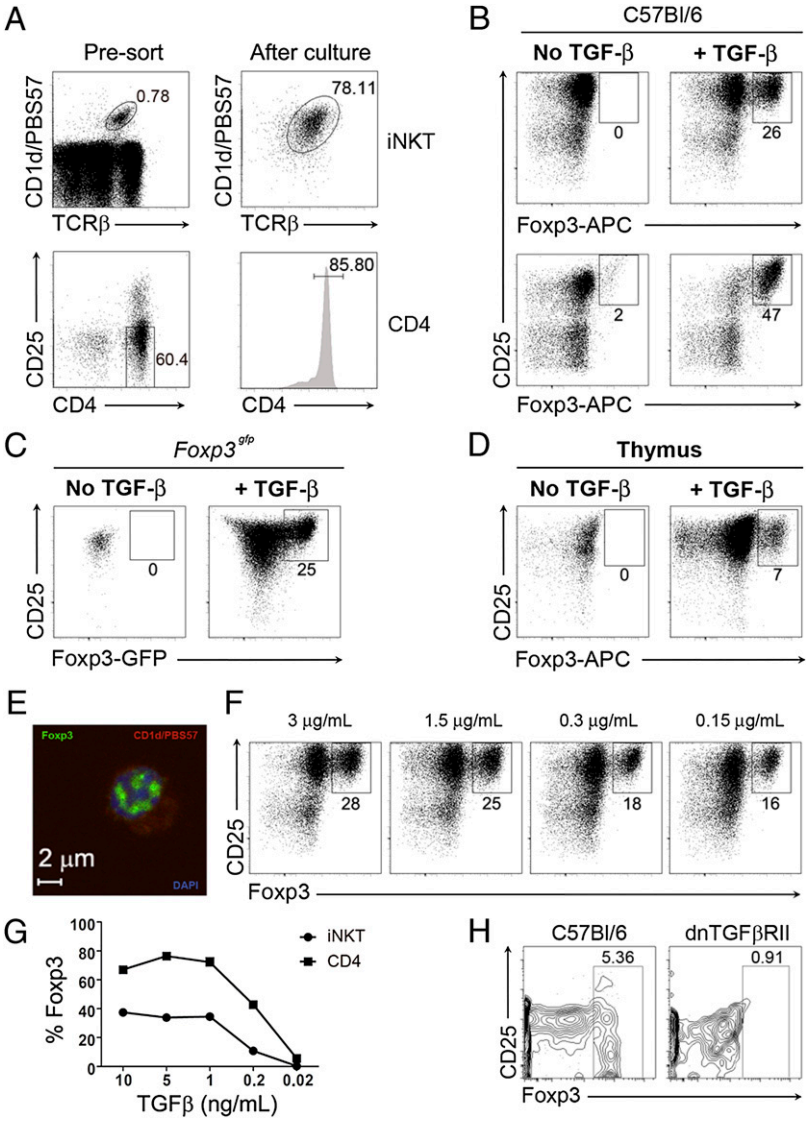

FIGURE 2. iNKT cells upregulate Foxp3 expression in the presence of TGF-β. *A–C*, Splenic iNKT cells and CD4$^+$CD25$^-$ T cells were sorted and stimulated with or without added TGF-β. *A*, Gating strategy used for cell sorting of iNKT cells (*upper panels*) and naive CD4 T cells (*lower panels*). The gating strategy used for analysis after 3 d of culture is also depicted for iNKT cells and CD4 T cells. *B*, Foxp3 expression in iNKT cells (*upper panels*) and control CD4 T cell cultures (*lower panels*) from C57BL/6 mice. *C*, Foxp3 expression in splenic iNKT cells from *Foxp3^{gfp}* knockin mice. *D*, Foxp3 expression in iNKT cells isolated from thymi of C57BL/6 mice. *E*, Foxp3 expression by iNKT cells was confirmed at single-cell level by confocal microscopy. Foxp3^{gfp}cells were FACS sorted after 3 d of culture, their invariant TCR was restained with PE-labeled CD1d/PBS57 tetramer (red), and the nucleus was counterstained with DAPI (blue). Foxp3 expression fluoresces in green (original magnification ×630). *F* and *G*, Titration of anti-CD3 or TGF-β concentration to address the impact on Foxp3 induction in splenic iNKT cells. *F*, iNKT cells cultured for 3 d in the presence of 5 ng/ml IL-2 and TGF-β, and stimulated with the indicated concentrations of anti-CD3. *G*, iNKT cells and CD4$^+$ CD25$^-$ T cells stimulated with 3 μg/ml anti-CD3 in the presence of 5 ng/ml of IL-2 and different concentrations of TGF-β after 3 d of culture. *H*, Foxp3 expression in iNKT cells from MLNs of C57BL/6 or dn TGFβRII mice following intragastric delivery of α-GalCer over 1 wk. Results are representative of three independent experiments with three mice per group.

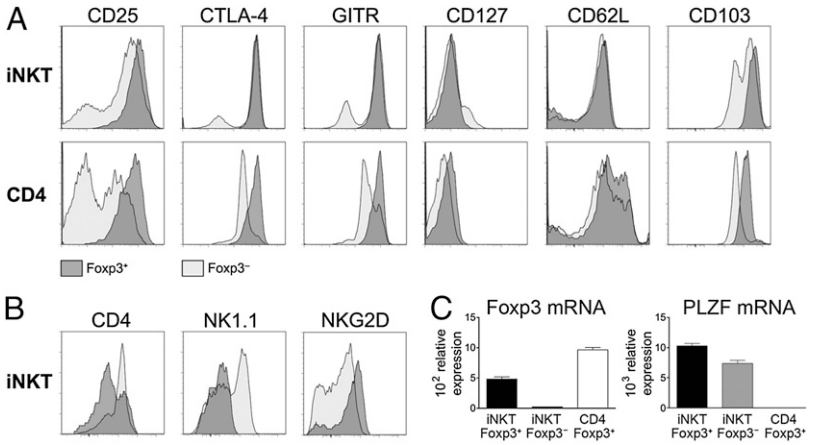

FIGURE 3. Phenotype of Foxp3$^+$ iNKT cells. *A* and *B*, iNKT cells and CD4$^+$CD25$^-$ T cells from the spleen of BALB/c (*A*) or C57BL/6 (*B*) mice were FACS sorted and cultured for 3 d in the presence of IL-2 and TGF-β. Histograms represent Foxp3$^+$ (gray) and Foxp3$^-$ (white) cells within iNKT or CD4 T cell subpopulations after conversion. Results are representative of two independent experiments. *C*, Quantification of Foxp3 and PLZF transcripts, relative to EF1A expression, from Foxp3-GFP$^+$ iNKT cells or CD4 T cells sorted after culture.

sion efficiency of iNKT cells when compared with CD4 T cell controls, iNKT cell numbers increased continuously over 14 d in culture.

We next tested the impact of TCR signal strength, costimulation, and cytokine addition on the conversion of iNKT cells into Foxp3 expressers. Maximal induction of Foxp3 was achieved with 3 μg/ml plate-bound anti-CD3 and 5 ng/ml TGF-β and IL-2, whereas further addition of IL-15 or IL-7 had little impact (Fig. 2*F*, Supplemental Fig. 4). Of note, titration of TGF-β concentration clearly revealed that this cytokine is essential for the induction of Foxp3 expression in iNKT cells (Fig. 2*G*).

TGF-β is required for expression of Foxp3 in iNKT cells in vivo

Th17 cell differentiation is critical for EAE development and requires a combination of cytokines, including TGF-β (24, 25). Accordingly, TGF-β neutralization at the time of EAE induction was shown to prevent the disease onset (26). Thus, to confirm TGF-β requirement for in vivo generation of Foxp3$^+$ iNKT cells, we adopted an alternative approach, taking advantage of the TGF-β–rich environment of the gut mucosa. We investigated whether intragastric delivery of α-GalCer could lead to the emergence of Foxp3$^+$ iNKT cells in mesenteric lymph nodes (MLNs), in the same way oral tolerance leads to de novo induction of Foxp3$^+$ Treg cells (27). We observed that α-GalCer delivery led to an accumulation of Foxp3$^+$ iNKT cells in MLNs (Fig. 2*H*). Of note, most Foxp3$^+$ iNKT cells from MLNs expressed low levels of CD25, something observed with the in vivo peripheral conversion of Treg cells in some conditions. To address whether in vivo generation of Foxp3$^+$ iNKT cells required TGF-β, we used dn TGFβRII mice (28), whose T and NKT cells are unable to transduce TGF-β signals, and observed no induction of Foxp3 expression in iNKT cells (Fig. 2*H*). Taken together, our observations suggest Foxp3$^+$ iNKT cells do not naturally arise during their development in the thymus, but can be induced in the periphery in environments where TGF-β is present.

Foxp3$^+$ iNKT cells display phenotypic characteristics of Treg cells and NKT cells

Once we established that iNKT lymphocytes could express Foxp3, we examined the phenotype of the converted cells. Many of the phenotypic characteristics of Foxp3$^+$ iNKT cells were shared with in vitro converted Foxp3$^+$ CD4 Treg cells. Both populations were predominantly CD25$^+$, CTLA-4$^+$, GITR$^+$, CD103$^+$, and IL-7Rα$^-$ (Fig. 3*A*). However, we also observed some differences between the two populations: Whereas Foxp3$^+$ CD4 T cells were heterogeneous for CD62L expression, Foxp3$^+$ iNKT cells were homogeneously CD62L^{low}. The absence of CD62L, in association with the high expression of CD103, suggests that in vivo Foxp3$^+$iNKT cells are excluded from lymph nodes and preferentially migrate to peripheral tissues. Interestingly, iNKT cells that had upregulated Foxp3 expression failed to secrete IL-4 and IFN-γ upon restimulation (Supplemental Fig. 6).

We also found that the capacity to induce Foxp3 expression was shared by CD4$^+$ and CD4$^-$ iNKT cells, as sorted CD4$^-$ and CD4$^+$ iNKT cells exhibited similar conversion efficiency (Fig. 3*B*) (M. Monteiro and C.F. Almeida, unpublished data). In addition, Foxp3$^+$ iNKT lymphocytes were NK1.1$^-$, and the majority expressed NKG2D. We also could detect the expression of PLZF, a transcription factor reported to distinguish NKT cells from conventional αβ T cells (10), in both sorted Foxp3$^+$ and Foxp3$^-$ iNKT cell subsets (Fig. 3*C*). Together, these observations indicate that induction of Foxp3 expression in iNKT lymphocytes does not corrupt their NKT cell nature.

Foxp3$^+$ iNKT cells migrate to the liver and maintain Foxp3 expression in vivo

To investigate the in vivo stability of Foxp3 expression in iNKT cells, we injected converted Foxp3-GFP$^+$ iNKT cells or control induced Foxp3-GFP$^+$ CD4 T (iTreg) cells into RAG2$^{-/-}$ mice (Fig. 4) or congenic Thy1.1 hosts (Supplemental Fig. 7). By contrast to iTreg cells, which migrated preferentially to the lymph nodes, but could also be found in the spleen and liver, 21 d after adoptive transfer iNKT cells could be detected only in the latter organ, where 50–80% (in empty hosts) or up to 10% (in wild-type congenic hosts) of iNKT cells maintained Foxp3 expression

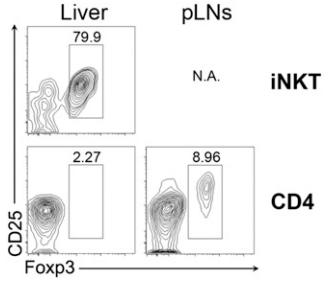

FIGURE 4. Foxp3$^+$ iNKT cells migrate to the liver. A total of 5×10^4 Foxp3-GFP$^+$ iNKT cells or CD4 T cells sorted from polarizing in vitro cultures were injected i.v. into RAG2$^{-/-}$ recipients. Data show Foxp3 and CD25 expression of iNKT cells and CD4 T cells, identified respectively as TCRβ intermediate, tetramer positive, and TCRβ$^+$CD4$^+$ cells inside the lymphocyte gate, in the liver, and in pLNs 21 d after adoptive transfer. iNKT cells were detected only in the liver. Data are representative of two independent experiments. pLNs, pooled lymph nodes.

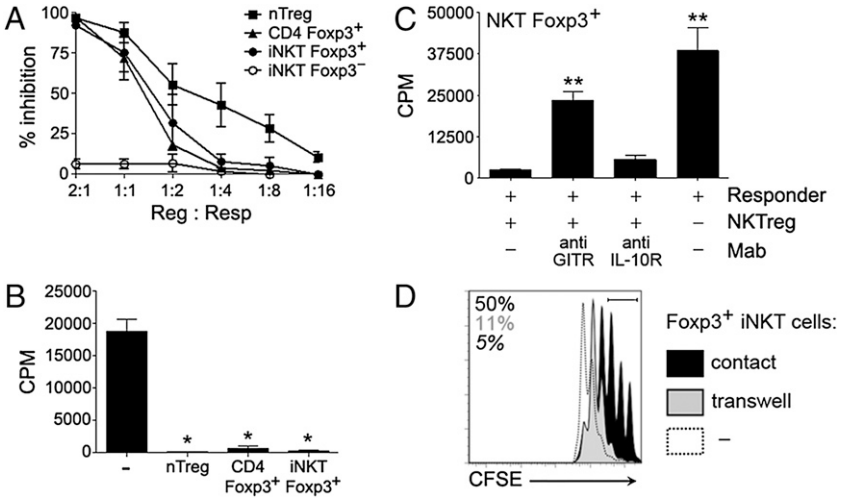

FIGURE 5. Foxp3$^+$ iNKT cells suppress T cell proliferation through a GITR-mediated contact-dependent mechanism. Foxp3$^+$ iNKT cells and Foxp3$^-$ iNKT cells sorted from the same cultures, iTreg cells (CD4 Foxp3$^+$), and nTreg cells were cocultured at different ratios with responder T cells. *A*, Average inhibition of proliferation from three independent experiments (each one with triplicates) normalized to proliferation of responder cells alone, assessed by thymidine incorporation. *B*, Representative experiment at a 2:1 ratio. $n = 3$; $*p < 0.05$. *C*, Addition of anti-GITR, but not anti–IL-10R, abrogated the suppressive effect of Foxp3$^+$ iNKT cells. $n = 4$; $**p < 0.01$. *D*, Proliferation of CFSE-labeled responder cells cultured for 72 h at 1:1 ratio with Foxp3$^+$ iNKT cells in a transwell assay, in which the two populations were cultured in contact (black histogram) or separated by a transmembrane (gray histogram). Dotted histogram shows proliferation in the absence of regulatory cells. *Upper left* numbers indicate the frequency of responder cells from the three conditions within the indicated gate.

(Fig. 4, Supplemental Fig. 7). In fact, it is becoming apparent that Foxp3 expression by conventional Treg cells is less stable than initially anticipated, especially under lymphopenic conditions (29). Our data indicate that in vivo Foxp3 stability in converted iNKT cells is not inferior to that of iTreg cells, although both populations have distinct homing potentials: Whereas CD4 Treg cells migrate predominantly to secondary lymphoid organs upon adoptive transfer, Foxp3$^+$ iNKT cells preferentially home to the liver. We thus suggest that the recruitment of regulatory iNKT cells to peripheral tissues may be important to complement and/or reinforce the local action of Treg cells (30).

Invariant regulatory NKT cells display contact-dependent GITR-mediated suppressive function

To evaluate the regulatory function of Foxp3$^+$ iNKT cells, we tested their ability to suppress proliferation of CD4$^+$CD25$^-$ "responder" cells. We used iNKT cells derived from *Foxp3^{gfp}* mice converted in the presence of TGF-β and, as controls, natural (nTreg) and iTreg cells. Titration of regulatory/responder cell ratio revealed that converted Foxp3$^+$ iNKT cells can indeed inhibit the proliferation of responder cells with similar efficiency to iTreg cells, and only slightly inferior to the efficiency of nTreg cells (Fig. 5*A*, 5*B*). Addition of anti-GITR (19), but not anti-IL-10R, neutralizing Abs to the cultures reversed suppression, indicating that GITR plays a predominant role in the regulatory function of Foxp3$^+$ iNKT lymphocytes (Fig. 5*C*). In agreement with these results, Foxp3$^+$ iNKT cells showed impaired suppressive effect compared with responder cell proliferation when cultured in a separate transwell (Fig. 5*D*), which excludes a major contribution for soluble factors to regulation. These results demonstrate that, similarly to conventional Treg cells (31, 32), induction of Foxp3 in iNKT lymphocytes endows these cells with suppressive function exerted through a contact-dependent

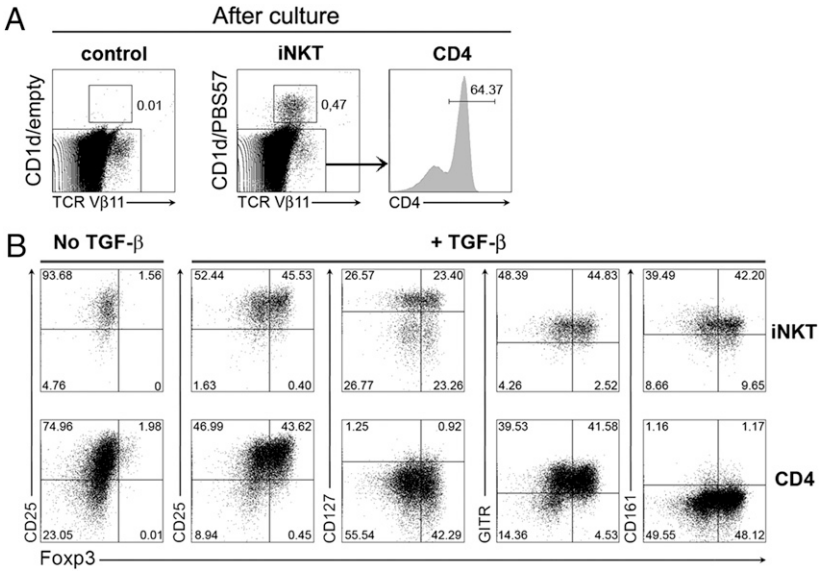

FIGURE 6. Foxp3 expression can be induced in human iNKT cells. Human iNKT cells and CD4$^+$ T cells from peripheral blood were magnetically enriched and cocultured for 5 d in the presence or absence of a conversion mixture, including or not including TGF-β. *A*, After culture, iNKT cells were identified by costaining of human CD1d/PBS57 tetramer and anti–TCR-Vβ11 Ab inside the lymphocyte gate (iNKT). Background of tetramer staining was evaluated with an empty human CD1d tetramer (control). CD4 T cells were gated inside the CD1d/PBS57-negative region (CD4). *B*, Flow cytometry data showing the coexpression of Foxp3 along with CD25, CD127, GITR, or CD161 in iNKT cell (*upper panels*) and CD4$^+$ T cell gates (*lower panels*). Results are representative of three independent experiments from different blood donors with at least three replicate cultures per condition.

mechanism mediated by GITR. This parallel with Foxp3$^+$ Treg cells prompted us to name these Foxp3$^+$ iNKT cells "Foxp3$^+$ invariant regulatory NKT (iNKTreg) cells".

TGF-β–mediated induction of Foxp3 expression in human iNKT cells

Finally, we also evaluated whether Foxp3 expression could be induced in human iNKT cells. Given the lower frequency of iNKT lymphocytes in human peripheral blood (31), we enriched total T cells by magnetic separation and cultured these bulk populations in polarizing conditions that included TGF-β and a mixture of neutralizing Abs against IL-12, IFN-γ, and IL-4 (32) (Fig. 6*A*). After 5 d of culture, up to 40% of human iNKT cells had upregulated Foxp3, an efficiency of conversion comparable to that of conventional CD4$^+$ T cells (Fig. 6*B*). The converted human Foxp3$^+$ NKT cells were CD25$^+$, GITR$^+$, and predominantly CD161$^+$, whereas CD127 was expressed by approximately half of the Foxp3$^+$ iNKT cells. Critically, these data establish that the induction of Foxp3 in NKT cells is a conserved phenomenon between rodents and humans.

Discussion

Among hematopoietic cells, Foxp3 expression has been claimed to be restricted to conventional T cells (24–27, 33). We now describe, for the first time, that iNKT and CD4 T lymphocyte populations share a similar competence in responding to a tolerogenic cytokine environment, namely, TGF-β, by expressing Foxp3 and undergoing functional specialization toward a regulatory phenotype. It should be noted, however, that Foxp3 induction upon activation in the presence of TGF-β is not a common feature of all lymphocytes: In particular, we were unable to convert sorted γδ T cells by CD3 stimulation in the presence of TGF-β (M. Monteiro and J. Ribot, unpublished observations). In addition, this potential appears to be a conserved feature of iNKT cells, as we were able to induce Foxp3 expression in samples from different murine strains and from human beings.

Our results clearly demonstrate that TGF-β is the critical cytokine promoting Foxp3 upregulation in iNKT cells, as described for CD4$^+$ T cell conversion into Foxp3$^+$ Treg cells (15–18). As a consequence, we took advantage of the TGF-β–rich environment of the gut to assess in vivo conversion of Foxp3$^+$ iNKT cells. In fact, it has already been described that oral delivery of an Ag can lead to a state of mucosal tolerance in which Ag-specific T cells are locally converted into Treg cells (27). In this paper, we show that intragastric delivery of α-GalCer induces the emergence of Foxp3$^+$ iNKTreg cells in MLNs. However, this process was impaired in mice with NKT cells unable to integrate the signals delivered through the TGF-βR, thus establishing a crucial role for TGF-β in the in vivo conversion of Foxp3$^+$ NKT cells. Therefore, although oral tolerance to proteic Ags, such as OVA, can be achieved in the absence of iNKT cells, as it has been shown using TCR-transgenic RAG-deficient mice (27), our results suggest that iNKT cells in the gut, exposed to glycolipid ligands, can convert into Foxp3$^+$ cells and synergize with local Treg cells in the promotion of tolerance to food Ags.

Most reports have shown that Foxp3$^+$ Treg cells suppress immune responses through cell contact-dependent mechanisms (34, 35). Several surface molecules preferentially expressed by Treg cells, such as GITR, have been implicated in their suppressive function (31, 32). We found that NKTreg cells can also suppress naive T cell proliferation by a GITR-dependent, IL-10–independent mechanism. Moreover, iNKTreg cells were unable to suppress responder cell proliferation when physically separated in a transwell, which excludes the additional contribution of soluble regulatory factors.

Although iNKT cells expressing Foxp3 have never been found in naive mice (21), the presence of this population has never been investigated in the context of an immune response. For instance, iNKT cells were reported to play important regulatory roles in the development of anterior chamber-associated immune deviation, transplantation tolerance, and prevention of autoimmunity, such as EAE, as lack of iNKT cells or decreased iNKT cell numbers compromise tolerance. However, in none of these studies was Foxp3 expression assessed in iNKT cells (33). Importantly, iNKT cell surface molecules largely overlap with those expressed by conventional CD4 T lymphocytes, and Foxp3$^+$ iNKT cells downregulate expression of NK1.1, a molecule often used in previous studies to distinguish NKT cells from T lymphocytes. Therefore, it is reasonable to assume that without an unambiguous identification with a specific tetramer, Foxp3$^+$ iNKT cells could have been erroneously detected as conventional Treg cells. Furthermore, until the development of a conditional knockout mouse lacking Foxp3 exclusively in iNKT cells—for instance, based on a PLZF-driven Cre expression system—it will not be possible to discriminate between the contribution of NKTreg cells and that of Treg cells in immune pathology. Although this topic surely deserves further investigation, the results presented in this paper clearly establish a new type of iNKT cell characterized by Foxp3 expression and immune regulatory properties similar to those of conventional Treg cells, one that might contribute to the maintenance of immune tolerance in the periphery.

Finally, the strong immunosuppressive properties displayed by NKTreg cells raise the possibility of their therapeutic application in the control of immune-mediated diseases, in particular, liver immune-mediated inflammation. Given their invariant specificity, Foxp3$^+$ NKTreg cells are assumed to have a general immunosuppressive action. It should be noted, however, that polyclonal Treg cell populations are likely to have, at least in part, a similar nonspecific immunosuppressive effect, possibly due to cross-reactivity with self-Ags (36). Indeed, these nonspecific effects can serve as the basis of Treg cell suppression in lymphopenia-driven proliferation and graft-versus-host disease (36–38). Notably, the invariant TCR expressed by Foxp3$^+$ iNKT cells might provide an advantageous, universally applicable method for their isolation, conversion, and expansion, regardless of genetic background or pathology.

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Disclosures

The authors have no financial conflicts of interest.

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